Faculdade de Medicina da Universidade do Porto

Protective effect of N-acetylcysteine (NAC) on human spermatozoa exposed to etoposide, a chemotherapeutic agent

Dissertação de Mestrado em Medicina e Oncologia Molecular

Ana Luísa Loureiro Rabaça

Orientador: Professora Doutora Rosália Sá, PhD

Departamento de Microscopia, Laboratório de Biologia Celular

Unidade Multidisciplinar de Investigação Biomédica (UMIB)

Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto

Coorientador: Doutor Pedro F. Oliveira, PhD

Departamento de Microscopia, Laboratório de Biologia Celular

Unidade Multidisciplinar de Investigação Biomédica (UMIB)

Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto

Porto 2015

Dissertação de candidatura ao grau de Mestre em Medicina e Oncologia Molecular submetida à Faculdade de Medicina da Universidade do Porto.

O presente trabalho foi desenvolvido no Instituto de Ciências Biomédicas Abel Salazar, sob a orientação científica da Professora Doutora Rosália Maria Pereira de Oliveira e Sá e coorientação do Doutor Carlos Pedro Fontes Oliveira.

Dissertation for applying to a Master's degree in Molecular Medicine and Oncology submitted to the Faculty of Medicine of the University of Porto.

The present work was developed at the Institute of Biomedical Sciences Abel Salazar, under the scientific supervision of Rosália Maria Pereira de Oliveira e Sá, PhD and co-supervision of Carlos Pedro Fontes Oliveira, PhD.

Ao meu Pai.

Publicações e Comunicações em Conferências

Do presente trabalho resultaram as seguintes publicações:

Publicações Internacionais:

Artigo de Revisão:

- **Rabaça A.**, Sousa M., Alves M. G., Oliveira P. F., Sá R. (2015) Fertility preservation in men undergoing chemotherapy: clinical relevance of protector agents. Current Medicinal Chemistry (aceite com revisões mínimas)

Artigo Prático de Investigação Original:

- **Rabaça A.**, Gonçalves A., Ferreira C., Barros A., Oliveira P. F., Sousa M., Alves M. G., Sá R. (2015) Chemotherapeutics and male fertility: N-Acetylscysteine Protects Human Sperm DNA integrity during Etoposide exposure. (submetido)

Publicações Nacionais:

- **Rabaça A.**, Ferreira C., Gonçalves A., Barros A., Alves M.G., Sousa M., Oliveira P.F., Sá R. (2015) N-acetilcisteína como agente protetor de espermatozoides expostos ao agente quimioterapêutico etoposido. *Revista Captar* (resumo)

Resultaram também as seguintes comunicações em conferências nacionais:

- **Rabaça A.**, Ferreira C., Gonçalves A., Barros A., Alves M.G., Sousa M., Oliveira P.F., Sá R. (2015) N-acetylcysteine: a protecting agent of spermatozoa against damage induced by the anticancer agent etoposide – III AEICBAS Biomedical Congress, 20 a 22 de março de 2015, Porto, Portugal – 2° lugar em "Melhor Apresentação de Poster – Medicina" (poster)

- **Rabaça A.**, Ferreira C., Gonçalves A., Barros A., Alves M.G., Sousa M., Oliveira P.F., Sá R. (2015) N-acetylcysteine as a protecting agent of spermatozoa against

damage induced by the anticancer agent etoposide – IV Encontro Nacional de Pós-graduação em Ciências Biológicas, 30 de março a 2 de abril de 2015, Aveiro, Portugal (poster nº 36)

- **Rabaça A.**, Ferreira C., Gonçalves A., Barros A., Alves M.G., Sousa M., Oliveira P.F., Sá R. (2015) Protective effects of N-acetylcysteine on human spermatozoa DNA during short-term etoposide exposure. UMIB Summit 2015, setembro de 2015 (poster aceite)

Agradecimentos

No decorrer deste ano foram várias as pessoas que me ajudaram a completar com sucesso mais uma fase da minha vida. A todos os que me acompanharam neste longo percurso quero desde já deixar o meu grande Obrigada! Agradeço em especial aos que, ao longo deste ano de dissertação, mais me orientaram, ajudaram e tornaram a conclusão desta etapa possível.

À **Professora Doutora Rosália Sá**, minha orientadora, agradeço tudo o que me ensinou neste ano. Agradeço toda a paciência, disponibilidade, amizade e apoio com que sempre me recebeu. Todos os seus ensinamentos científicos, correções, críticas, sugestões e conselhos foram essenciais para a minha formação e sem eles este trabalho não teria sido possível. Obrigada por me ter ajudado a vencer este desafio. Muito Obrigada!

Ao **Doutor Pedro Oliveira**, meu coorientador, agradeço toda a simpatia e disponibilidade ao longo deste ano. Agradeço todas as correções e sugestões que permitiram o desenvolvimento deste trabalho científico.

Agradeço também ao **Instituto de Ciências Biomédicas Abel Salazar – ICBAS** por me ter acolhido ao longo da minha dissertação e me ter proporcionado todas as condições laboratoriais necessárias para o desenvolvimento deste projeto.

À **Faculdade de Medicina da Universidade do Porto – FMUP**, em especial ao **Mestrado em Medicina e Oncologia Molecular**, na pessoa do **Professor Doutor Henrique de Almeida**, queria agradecer a oportunidade que me proporcionou. Foi uma fase bastante gratificante da minha vida, repleta de novos conhecimentos e com um enorme desenvolvimento pessoal. Agradeço também todo o financiamento disponibilizado para a realização desta dissertação, sem ele tudo isto não teria sido possível.

Ao **Professor Doutor Mário Sousa** agradeço a oportunidade que me proporcionou ao aceitar-me no seu grupo. Muito obrigada por me ter permitido trabalhar nesta área, foi, sem dúvida, um ano em que aprendi imenso.

Ao **Doutor Marco Alves** agradeço toda a disponibilidade demonstrada ao longo deste ano. As suas críticas e sugestões foram fundamentais para a conclusão com sucesso deste projeto.

Ao **Professor Doutor Alberto Barros**, diretor do Centro de Genética da Reprodução Prof. Alberto Barros, agradeço toda a sua colaboração, sem a qual o desenvolvimento deste projeto não teria sido possível. Agradeço também a todos os colaboradores da clínica pela disponibilidade e simpatia com que sempre me receberam. Agradeço em especial à **Dra. Ana Gonçalves** por toda a sua dedicação a este projeto.

A todas as pessoas do Laboratório de Biologia Celular, em especial à **Dona Elsa** e à **Ângela**, agradeço toda a simpatia e amizade com que me receberam. Estiveram sempre disponíveis para tudo o que foi necessário ao longo deste ano e toda a sua paciência, ensinamentos e conselhos foram essenciais.

Aos colegas de laboratório **Raquel, Tito, Ana, Gustavo, Tânia, Maria João e Susana** agradeço todos os ensinamentos, simpatia e companheirismo. Todo o tempo de trabalho no laboratório, os almoços, jantares e inúmeras conversas. Obrigada.

À **Carolina**, a minha companheira de laboratório, agradeço toda a amizade, companhia e paciência ao longo deste ano. Foi um caminho que percorremos juntas! Obrigada por todas as horas que passamos juntas no laboratório ou na biblioteca. Obrigada por teres sido em muitos momentos a minha confidente, me teres compreendido como ninguém, me teres dado incentivo e força. Sem ti este ano não teria de todo sido o mesmo. Muito Obrigada!

Aos **AMIGOS** de sempre, a **Malta do Costume**, aqueles que nunca deixaram de estar ao meu lado, queria deixar o meu enorme Obrigada! A vossa paciência é infinita, e todo o incentivo e força que me deram foram essenciais ao longo deste ano. Sem vocês este percurso não teria sido o mesmo. Obrigada por todas as vezes que me ouviram e me deram palavras de ânimo e conselhos. Obrigada por todos os jantares, saídas, passeios e momentos de puro ócio que me permitiram sorrir e recarregar baterias. Obrigada por terem estado sempre lá!

Ao **Pedro** agradeço todo o amor, carinho, compreensão e dedicação. Agradeço todas as nossas infindáveis conversas e toda a tua paciência. Obrigada por nunca teres duvidado de mim. Obrigada por toda a tua ajuda, pelos teus conselhos e incentivo. Obrigada por me animares mesmo nos piores momentos. Obrigada por sempre me teres acompanhado e vibrares tanto com os meus sucessos quanto eu. Contigo ao meu lado nada parece impossível. Muito Obrigada!

À minha **Madrinha** agradeço todo o amor, carinho e dedicação. Sempre foste como uma segunda mãe para mim e eu espero encher-te sempre de orgulho. Obrigada por tudo o que fizeste por mim ao longo deste ano e ao longo da minha vida! Obrigada por estares ao meu lado a torcer por mim na conclusão de mais uma etapa da minha vida.

À minha **Mãe** agradeço esta oportunidade. Sem ti nada disto seria possível. Tentei estar à altura das tuas expectativas e espero que te orgulhes sempre de mim. Obrigada por todo o teu amor e sacrifícios. A vida é matreira mas nós perseveramos. Obrigada por me incentivares a nunca desistir dos meus sonhos e a lutar por aquilo em que acredito. Obrigada por acreditares sempre em mim! Obrigada por tudo!

Resumo

As neoplasias malignas têm vindo a afetar um número crescente de crianças, adolescentes e homens em idade reprodutiva. O potencial reprodutivo destes pacientes é negativamente afetado pelos regimes quimioterápicos adotados, existindo o risco de estes virem a sofrer de infertilidade prolongada. O etoposido é um composto quimioterápico citotóxico utilizado no tratamento de diversas neoplasias. É um agente semissintético, da classe dos inibidores da topoisomerase II, que aumenta o número de complexos de clivagem do DNA, provocando quebras permanentes na dupla cadeia. Além de atuar diretamente no DNA, este composto poderá causar danos celulares através da indução de stress oxidativo. O etoposido afeta a espermatogénese, e a recuperação da fertilidade após a exposição a este agente está associada com a dose utilizada e a duração do tratamento, sendo assim difícil de prever. Portanto, existe uma forte necessidade de identificar um composto capaz de preservar a fertilidade masculina durante a exposição a etoposido, sem interferir com a eficácia do tratamento. O N-acetilcisteína (NAC), um percursor da L-cisteína, possui propriedades antioxidantes e quimioprotetoras. Anteriormente foi demonstrado que este composto é seguro para uso clínico e tem vários efeitos protetores nas células. Além disso, estudos indicaram que o NAC é capaz de melhorar os parâmetros seminais e proteger as células de Sertoli de danos induzidos pelo stress oxidativo. Como foi relatado que o NAC possui propriedades citoprotetoras, o presente trabalho teve como objetivo avaliar o seu potencial como preservador da qualidade espermática durante a exposição dos espermatozoides humanos a etoposido.

No nosso estudo, espermatozoides humanos foram incubados durante 2 h a 37°C com 25 µg/ml de etoposido, 50 µM de NAC ou uma combinação de ambos os compostos. Após incubação, procedeu-se à avaliação dos parâmetros seminais, tais como motilidade, morfologia e vitalidade, bem como à análise da fragmentação de DNA e condensação da cromatina. Foram também medidos os danos oxidativos e avaliado o metabolismo dos espermatozoides por espectroscopia de ressonância magnética nuclear de protões (^{1}H-RMN). Os nossos resultados demonstram que a exposição a curto prazo ao etoposido provoca alterações na cromatina e aumenta a fragmentação do DNA dos espermatozoides. Além disso, os resultados obtidos mostram que o etoposido não induz danos oxidativos nem causa alterações no perfil glicolítico dos espermatozoides. A adição de NAC aos espermatozoides expostos a etoposido preserva a condensação da cromatina, e reduz a fragmentação do DNA.

Assim, embora a exposição a etoposido por um curto prazo não afete a vitalidade, é capaz de induzir graves alterações na cromatina, e causar danos no DNA dos

espermatozoides. Além de estas alterações puderem comprometer a fertilidade masculina, são potencialmente transmissíveis à próxima geração. A adição de NAC aos espermatozoides expostos a etoposido protege a integridade da cromatina e reduz a fragmentação do DNA. Deste modo, o NAC atua como um agente citoprotetor, protegendo os espermatozoides dos danos no DNA induzidos pelo etoposido. Contudo, esta exposição a curto prazo não induz danos oxidativos nem provoca alterações no perfil glicolítico destas células, sugerindo que este quimioterápico afeta diretamente o DNA dos espermatozoides. A capacidade do NAC para proteger os espermatozoides humanos dos danos de DNA induzidos pelo etoposido pode ser de relevância clínica. A maioria dos pacientes submetidos a quimioterapia não procede à colheita de amostras de sémen antes do início do tratamento, pelo que a integridade dos espermatozoides recolhidos após o início do tratamento com etoposido pode ser preservada com a adição de NAC. Assegurando assim, que a maioria das células viáveis teria a integridade de DNA preservada e poderia ser utilizada com segurança em futuras técnicas de Reprodução Medicamente Assistida. No entanto, apesar destes resultados promissores, mais estudos devem ser realizados para confirmar a eficácia *in vivo* destes resultados e identificar uma terapia complementar eficaz que seja capaz de proteger a fertilidade masculina dos efeitos secundários causados pela quimioterapia. Até que tal terapia esteja disponível, é fundamental que todas as opções de preservação da fertilidade masculina sejam discutidas com os pacientes antes do tratamento ser iniciado, para assim permitir uma futura paternidade.

Palavras-chave: Antioxidante; Espermatozoides; Etoposido; Infertilidade masculina; NAC; Quimioterapia.

Abstract

Cancer has been affecting a growing number of children, adolescents and adult males in reproductive age. Male reproductive potential is adversely affected by chemotherapeutic drugs and patients are at risk for prolonged infertility. Etoposide is a chemotherapeutic drug used in the treatment of several neoplasia. It is a semi-synthetic agent, which acts via topoisomerase II inhibition, highly increasing the number of covalent DNA cleavage complexes, what results in permanent double-stranded breaks. Besides its interaction with topoisomerase II, it may also cause DNA damage by oxidative stress induction. Etoposide is known to affect spermatogenesis, and fertility recovery after drug exposure is associated with the dose used and treatment length, thus being difficult to predict. As a result, there is a strong need to identify a compound able to preserve male fertility during etoposide exposure without interfering with the efficacy of treatment. N-acetylcysteine (NAC), an L-cysteine precursor, has chemopreventive and antioxidant properties. It has been demonstrated that this agent is safe for clinical use and has several protective effects on cells. Furthermore, studies have shown that NAC improves semen parameters and protects Sertoli cells from damage induced by oxidative stress due to its antioxidant potential. As NAC has been reported to possess cytoprotector properties, we hypothesized that it may be a good preserver of sperm quality during short-term etoposide exposure.

In our experiments, human spermatozoa were incubated for 2 h at 37 ºC with 25 µg/ml of etoposide, 50 µM of NAC and both drugs in combination. Spermatozoa parameters, such as motility, vitality and morphology, as well as DNA fragmentation and chromatin condensation were evaluated. Oxidative damages were measured and sperm metabolism was studied by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). Our results demonstrate that short-term exposure to etoposide induces chromatin alterations and DNA fragmentation. Moreover, etoposide does not induce sperm oxidative damages nor glycolytic profile alterations. The addition of NAC to sperm exposed to etoposide preserved sperm morphology and chromatin condensation and reduced sperm DNA fragmentation.

Thus, although short-term exposure to etoposide does not affect sperm vitality, it induces severe chromatin alterations and DNA damages to human spermatozoa. Besides these alterations may compromise male fertility, being potentially transmittable to the next generation. The addition of NAC to sperm exposed to etoposide protected cellular chromatin integrity and reduced DNA fragmentation. Therefore, it acts as a cytoprotector agent, shielding human sperm DNA from etoposide-induced damages. A short-term exposure of human spermatozoa to etoposide does not induce cellular oxidative damages nor glycolytic profile alterations, providing evidence that etoposide directly affects sperm

DNA. NAC's ability to preserve human spermatozoa from DNA damages induced after a short-term exposure to etoposide may be of clinical relevance. The majority of patients undergoing chemotherapy fail to collect semen samples prior to the initiation of treatment, therefore, the integrity of the collected spermatozoa after etoposide treatment initiation could be preserved with the addition of NAC, assuring that the majority of viable cells would have their DNA integrity preserved and could be safely used in Medically Assisted Procreation. Although results are promising, further research must be implemented to identify an effective supplemental therapy that addresses the multiple side effects of chemotherapy on male reproductive function. Until such therapy is available, it is fundamental that all fertility preservation options are discussed with patients, before treatment is initiated, to allow parenthood.

Key-words: Antioxidant; Chemotherapy; Etoposide; Male infertility; NAC; Spermatozoa.

Table of contents

List of tables

List of figures

List of abbreviations

μg – Microgram

μl – Microliter

^{1}H-NMR – Proton Nuclear Magnetic Resonance

3-NT – 3-nitrotyrosine

4-HNE – 4-Hydroxynonenal

5-FU – 5-fluorouracil

6-MP – 6-mercaptopurine

AB – Aniline blue

Ara-C – Cytarabine

AS101 – Trichloro, (dioxoethylene-O, O') tellurite

AST – Astaxanthin

BCNU – Carmustine

CCNU – Lomustine

CNPMA – National Council on Medically Assisted Procreation

DAPI – 4′,6-diamidino-2-phenylindole

DNA – Deoxyribonucleic acid

DNP – 2,4- dinitrophenyl

DNPH – 2,4-dinitrophenylhydrazine

DPPD – 1-N, 4-N-diphenylbenzene-1, 4-diamine

EA – Ellagic acid

FSH – Follicle-stimulating hormone

GnRH-A – Gonadotropin-releasing hormone antagonist

GSH – Glutathione

HO$^{\bullet}$ – Hydroxyl radical

LA – α-lipoic acid

mM – Milimolar

MTX – Methotrexate

NAC – N-acetylcysteine

ºC – Celsius degrees

PBS – Phosphate Buffered Saline

PMA – Medically Assisted Procreation

PVDF – Polyvinylidene Difluoride

RJ – Royal jelly

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Room Temperature

sDNAfrag – Sperm DNA fragmentation

SPM – Sperm Preparation Medium

TUNEL – Terminal doxynucleotidyl tranferase dUTP nick-end labelling

VBL – Vinblastine

VCR – Vincristine

WHO – World Health Organization

I. Introduction

Article I: Novel drug therapies for fertility preservation in men undergoing chemotherapy: clinical relevance of protector agents

Article I:

Novel drug therapies for fertility preservation in men undergoing chemotherapy: clinical relevance of protector agents

A. Rabaça[a,b], M. Sousa[a,c], M.G. Alves[d], P.F. Oliveira[a] , R. Sá[a,*]

[a]Department of Microscopy, Laboratory of Cell Biology, Institute of Biomedical Sciences Abel Salazar (ICBAS), Unit for Multidisciplinary Research in Biomedicine (UMIB), University of Porto, Portugal; [b]Faculty of Medicine, University of Porto (FMUP), Porto, Portugal; [c]Centre for Reproductive Genetics Prof. Alberto Barros, Av. do Bessa 240, 1º Dto. Frente, 4100-009 Porto, Portugal; [d]Health Sciences Research Centre (CICS), University of Beira Interior (UBI), Covilhã, Portugal

Abstract: Cancer has been affecting a growing number of children, adolescents and adult males in reproductive age. Male reproductive potential is adversely affected by chemotherapeutic drugs and patients are at risk for prolonged infertility. Fertility recovery is related to the chemotherapeutic agent and dosage used, being thus difficult to predict. As a result, there is a strong need to identify a natural or synthetic compound that is able to preserve male fertility without interfering with the efficacy of the chemotherapeutic regimen. New procedures, as well as several drugs, are being investigated to assess their efficiency in protecting male reproductive functions from the chemotherapy side-effects. This review provides an overview of the wide range of chemotherapeutic drugs regularly used in cancer treatment and their detrimental effects on male fertility. In addition, it also assesses the existing protector agents for male fertility and their usefulness in preserving and protecting male reproductive functions exposed to chemotherapeutics. Several protector agents for male fertility are being studied, and results are promising. Nonetheless, further research must be implemented to identify a supplemental therapy that addresses the multiple side effects of chemotherapy on male reproductive function. Until such therapy is discovered, it is fundamental that all fertility preservation options are discussed with patients, before treatment is initiated, to assure parenthood.

Keywords: male infertility; chemotherapy drugs; cytoprotector agents; antioxidants; hormone therapy.

1. Introduction

The global cancer burden has been increasing over the last decades. There is striking evidence of a growth in cancer incidence among children and adolescents. In fact, approximately 15% of male cancer patients are 55 years of age or younger, and 25% of those are still below their twenties (*1*). Chemotherapy and radiotherapy regimens allow the survival of a higher number of patients and, these days, the majority of children and adolescents who have been diagnosed with cancer can truthfully hope for long-term survival (*2-4*). With increasing survival rates, the concern with the life quality of the surviving patients is a crucial issue (*5*). The cytotoxic chemotherapy is associated with substantial morbidity (*6*). The testes are highly vulnerable to chemotherapy toxicity and hence, alterations in gonadal functions that might impair male fertility are one of the most common treatment side effects (*5, 6*). Chemotherapeutic drugs target preferentially the cellular cycle, thus being highly detrimental for male spermatogenesis, damaging the germinal epithelium and decreasing sperm quality (*7*).

Spermatogenesis is a complex biological process that begins in the testes of pubertal men. It comprehends the entire development course of a diploid stem cell, the spermatogonium, to a very specialized haploid cell with unique characteristics, the spermatozoa. This process occurs within the seminiferous tubules for a length of around 70 days and produces approximately 70 million spermatozoa daily (*8*). Spermatogonia are located on the basal membrane of the seminiferous tubules and are the precursors of all male germ cell types. These cells are responsible for the initiation of the spermatogenesis process, undergo continuous self-renewal by mitosis and regulate cell population ensuring an appropriate balance between germ cells and Sertoli cells (*9*). Spermatogonia can be divided into two different types, A and B, depending on their heterochromatin content. Type B spermatogonia have a higher amount of heterochromatin, an indication of a differentiating cell, and are consequently the precursors of spermatocytes (*8-10*). The spermatocytes cross the blood-testis barrier, formed by the Sertoli cells tight junctions, and go through meiosis. During the course of meiosis there is an exchange of genetic material between homologous chromosomes that then migrate to separate cell poles and give origin to secondary spermatocytes at the end of meiosis I. Then these secondary cells undergo the second phase of meiosis forming four haploid round spermatids (*11*). Once meiosis is completed, spermatids experience spermiogenesis, differentiating into elongated, condensed, mature spermatozoa, that are released in the lumen of the seminiferous tubules (*8*).

Although spermatogenesis is maintained throughout life, cytotoxic chemotherapy and radiotherapy may cause persistent damage and lead to transient or persistent oligospermia

and azoospermia (*12, 13*). Fertility recovery is related to the chemotherapeutic drug taken and the dosage used, thus being very difficult to predict. In fact, up to 30% of juvenile cancer survivors persist sterile for the remaining of their lives (*14*). For that reason efforts have been made to discover new approaches to maintain the reproductive health of such patients. However, to this day, there is no pharmacological protocol to prevent or diminish the damage induced by chemotherapy on spermatogenesis (*15*).

Semen cryopreservation is so far the most common approach to face this problem. Even though it offers patients a chance to preserve their fertility, it presents some limitations. Firstly, pre-pubertal boys are unable to produce mature spermatozoa and although testicular tissue of the undeveloped testes contains spermatogonial stem cells that have been able to re-establish fertility after transplantation in mammals (*16*), further technological development is still required before it can be applied to humans (*17, 18*). In addition, semen parameters in cancer patients are often altered even before the initiation of treatment (*19*). Moreover, in order to achieve the best results possible, cryopreservation must be performed before the chemotherapeutic regimen is initiated and several samples should be preserved (*18*). Nonetheless, studies have shown that oncologists and their patients frequently do not consider this option until therapy has begun (*20*). Consequently, efforts have been made to determine new procedures that are capable of protecting spermatogenesis and spermatozoa from the adverse effects of chemotherapy (*6, 12*).

2. Adverse effects of chemotherapeutic drugs on male fertility

Chemotherapeutic drugs target mainly dividing cells, being very detrimental for male spermatogenesis (Figure I-1). These agents can easily reach the proliferative epithelium of the seminiferous tubules and some can even infiltrate the blood-testis barrier and harm the later stages of differentiating germ cells, which are very susceptible to cytotoxic agents (*21*). Gonadotoxic therapies are used not only in cancer treatment but in other disorders, such as autoimmune diseases, myelodysplatic syndromes and bone marrow transplants (*7, 22*). The risk of infertility due to these treatments is related to the drug, dose and length of period for which the drug is used (*3*). The chemotherapeutic drugs used in the clinical practice can be divided into different categories according to their origin and means of action.

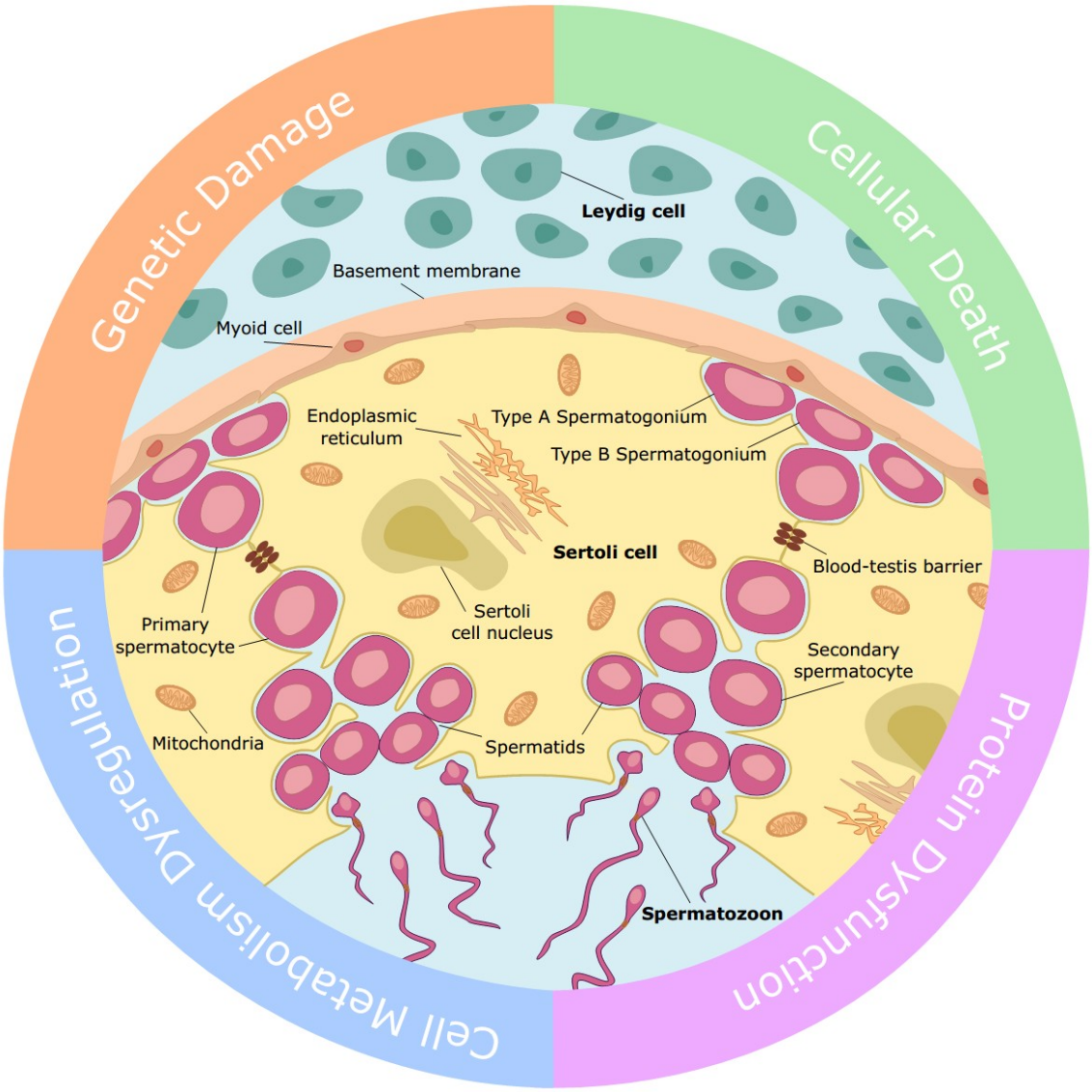

Figure I-1. Schematic illustration of spermatogenesis and the detrimental effects triggered by chemotherapeutic drugs. Sertoli and developing germ cells compose the seminiferous epithelium while Leydig and myoid cells are located in the interstitial space. Spermatogenesis is the process in which the male gametes, spermatozoa, are produced. Sertoli cells constitute the physical and nutritional support of the developing germ cells. Type A spermatogonium divides and develops into type B spermatogonium, which enters meiotic prophase and differentiates into primary spermatocyte which in turn undergoes meiosis I and forms the secondary spermatocyte. After meiosis II is complete, four haploid spermatids migrate toward the lumen, where fully formed spermatozoa are finally released. Chemotherapeutic agents can disrupt spermatogenesis by directly interacting with testicular cells, causing cellular metabolism dysregulation, protein dysfunction, genetic damages and cellular death.

2.1 Alkylating Agents

The first chemotherapeutic agent to obtain approval for use in the clinical practice was an alkylating drug. Alkylating agents are electrophilic elements that interact with the functional group of nuclear proteins or DNA, covalently transferring alkyl groups (C_nH_{2n+1}).

Consequently, alkylating agents lead to protein inactivation or cytotoxic DNA strand breaks (*42-44*). They are used in the treatment of diverse cancers types, like lymphoma, Hodgkin disease, non-Hodgkin's lymphoma, leukaemia and sarcomas (*42*). These chemotherapeutic agents are highly detrimental to cells and carry numerous side effects on male fertility (Table I-1).

Table I-1. Alkylating agents and their detrimental effects on male fertility

Chemotherapeutic drug	Formula	Effect on male fertility	Infertility Risk	Study Type	Species
Chlorethamine	$C_5H_{11}Cl_2N$	Prolonged azoospermia Reduced sperm count and motility	High	*In Vivo*	Human (*23, 24*)
Chlorambucil	$C_{14}H_{19}Cl_2NO_2$	Prolonged azoospermia	High	*In Vivo*	Mouse (*25, 26*)
Cyclophosphamide	$C_7H_{15}Cl_2N_2O_2P$	Prolonged decrease in sperm count or azoospermia	High	*In Vivo* *In Vitro*	Mouse (*27*) Human (*28*) Rat (*29*)
Ifosfamide	$C_7H_{15}Cl_2N_2O_2P$	Prolonged azoospermia	High	*In Vivo*	Rabbit (*30*) Human (*31*)
Busulfan	$C_6H_{14}O_6S_2$	Damage stem and differentiating spermatogonia Likely to cause prolonged azoospermia	High	*In Vivo*	Mouse (*32, 33*)
Carmustine (BCNU)	$C_5H_9Cl_2N_3O_2$	Azoospermia after pre-pubertal treatment	High	*In Vivo*	Mouse (*34-36*) Rat (*37*)
Lomustine (CCNU)	$C_9H_{16}ClN_3O_2$	Damage differentiating spermatogonia	High	*In Vivo*	Mouse (*34, 36*)
Carboplatin	$C_6H_{12}N_2O_4Pt$	Damage spermatogonia, Sertoli cells and spermatozoa membranes Temporary azoospermia	Moderate	*In Vivo* *In Vitro*	Mouse (*38*) Rat (*39*) Human (*40*) Human (*41*)

Chlorethamine and chlorambucil are two alkylating agents that belong to the group of nitrogen mustards, which present a high risk for prolonged azoospermia after treatment. Chlorethamine is typically used in combination therapies for the treatment of Hodgkin disease and non-Hodgkin's lymphoma. Studies have shown that therapies employing chlorethamine as the main agent exhibited up to 97% of azoospermia after treatment and, for the majority of the patients, the recovery rate of spermatogenesis was slow, with a decade of spermatogenesis hiatus (*23, 24*). Furthermore, chlorethamine regimens have shown to decrease sperm concentration and by the end of patient follow-up there was still

no evidence of motile spermatozoa (*23*). Chlorambucil is an antineoplastic and immunosuppressive agent mainly used in the treatment of lymphomas and immunological disorders (*26*). An *in vivo* study in mice treated with chlorambucil has shown that these agent induced heritable mutations in germ cells, especially in early spermatids (*25*). Chlorambucil is very detrimental for spermatogenesis being highly cytotoxic for male spermatogonia and capable of inducing chromosomal aberrations in spermatids and spermatozoa, which leads to a prolonged state of azoospermia after treatment (*26*).

Cyclophosphamide and ifosfamide are synthetic forms of nitrogen mustards that in comparison to their precursors are more stable and less toxic to cells. These two pro-drugs are normally used in the treatment of various solid tumours and sarcomas. However, they have a wide range of side effects, including prolonged azoospermia, especially thanks to their metabolites (*42*). Studies conducted in mice that underwent different cycles of cyclophosphamide treatment showed that this drug is responsible for a decrease in all the differentiated cells of the testis (*27*). In male rats, it was demonstrated that germ cells with DNA damage, due to cyclophosphamide exposure, pursued spermatogenesis and produced damaged spermatozoa, which may affect the pregnancy success rate (*29*). In humans, it has been described that cumulative doses of cyclophosphamide are more likely to cause long term sterility than a single dose treatment (*28*). Ifosfamide is mainly used in combination with other antineoplastic agents. Nonetheless, a study with rabbits assessed the effects of ifosfamide, as a single agent, on male fertility. This study revealed that varying dosages of ifosfamide cause transient and dose-dependent oligospermia (60, 90, 120, and 240 mg/kg), teratozoospermia (120 and 240 mg/kg) and asthenozoospermia (240 mg/kg). Furthermore, spermiogenesis was decreased with dosages of 60, 90, and 120 mg/kg of ifosfamide, while spermatocytogenesis and sperm maturation in the epididymis were severely affected at the highest dosage tested (240 mg/kg) (*30*). Ishikawa and colleagues evaluated cancer patients treated with a high-dose chemotherapeutic regimen, where 7.5 g/m^2 of ifosfamide was used in combination with other agents. Their results showed that although the majority of patients exhibited spermatogenesis arrest during treatment and were considered azoospermic, in the follow up 50% of the men were normospermic (*31*).

Busulfan is the only alkyl alkane sulfonate used in the medical field. It is an antineoplastic and immunosuppressive agent with myeloablative properties mostly used for allogeneic hematopoietic progenitor cell transplantation in patients with chronic myeloid leukaemia (*42*). The cytotoxic activity of this drug is attributable to its ability to induce DNA-DNA and DNA-protein cross links. A study on mice exposed to bulsufan has demonstrated that it is able to accelerate the germ cell apoptotic rate, and that a single dose of 40 mg/kg

is capable of completely abolish the male germinal epithelium (*33*). In fact, with doses this high, mice became permanently sterile (*32*).

The group of nitrosoureas alkylating agents, of which carmustine (BCNU) and lomustine (CCNU) are part of, are very effective anti-proliferative drugs that have the ability to cross the hemato-encephalic barrier (*42, 45*). Hence, these agents are primarily used in the treatment of brain tumours, but are also used to treat melanomas and some carcinomas (*42*). BCNU and CCNU are agents with elevated gonadotoxicity and sterilizing capability, both in pre-pubertal boys and adults. These two drugs are responsible for the induction of mutations throughout all stages of spermatogenesis in a dose dependent manner (*36*). When administered at high doses (500 mg/m^2 to 1 g/m^2) they cause azoospermia, while in lower dosages they are usually given as part of a combined therapy with other highly sterilizing agents (*46*). Studies have shown that BCNU not only affects the spermatogonia, but is also capable of crossing the blood-testis barrier reaching spermatocytes and spermatids (*34*). Tates and collaborators described that BCNU is able to, in addition to producing chromatid aberrations in spermatogonia, cause translocations in spermatocytes (*35*). Research on rat models also revealed that, although female reproductive capacity is not affected by BCNU, males presented a disrupted reproductive aptitude during and after treatment. Moreover, a high percentage of male rats had ceased spermatogenesis and the frequency of embryonic loss was elevated (*37*).

Carboplatin is a platinum analogue frequently used in chemotherapeutic regimens of neoplasia that affect young people and children. This chemical is highly stable, which confers a lower reactivity with DNA, thereby being less cytotoxic (*47*). However, a superior dose is required to obtain binding to DNA and effective antineoplastic activity (*48*). The effects of carboplatin on male mice testes have been investigated by Kopf-Maier and peers, which discovered that this agent affects fertility. Even though it is less reactive with DNA, the high doses needed to obtain the desired antineoplastic effects make it as toxic to cells as other commonly used alkylating drugs (*38*). Fuse and peers obtained similar results on male rats with carboplatin being harmful to spermatogenesis, specially affecting spermatogonia and Sertoli cells (*39*). Reiter and peers performed an *in vitro* study with human spermatozoa and found that carboplatin is responsible for significant damage on sperm membrane integrity (*41*). However, humans exposed to carboplatin present a high percentage of spermatogenesis recovery after treatment (*40*).

2.2 Cytotoxic Antibiotics

Cytotoxic antibiotics are drugs of microbial origin highly effective as antineoplastic agents. They are non-phase-cycle specific and act by interfering with DNA replication

causing the arrest of the cell cycle. However, their complete mechanism of action is still not completely understood (*49, 50*). The information regarding the toxicity mechanism of these antibiotics is divisive. Some studies suggest these agents form free radicals in cells, induce DNA damage and enhance lipid peroxidation, while others found contradictory results (*51*). Despite all these uncertainties, there are several pathologies were these agents are prescribed, like lymphomas, leukaemia, sarcomas and testicular carcinomas (*49-51*). In Table I-2 we have summarized cytotoxic antibiotics and their risks for male fertility.

Table I-2. Cytotoxic antibiotics and their detrimental effects on male fertility

Chemotherapeutic drug	Formula	Effect on male fertility	Infertility Risk	Study Type	Species
Doxorubicin	$C_{27}H_{29}NO_{11}$	Killed stem and differentiating spermatogonia Reduced sperm count and motility	Moderate	*In Vivo*	Mouse (*52*) Rat (*53, 54*)
Dactinomycin	$C_{62}H_{86}N_{12}O_{16}$	Damaged spermatogonia and spermatids Reduced sperm count	Low	*In Vivo*	Mouse (*34*) Human (*55*)
Bleomycin	$C_{55}H_{84}N_{17}O_{21}S_3^+$	Reduced sperm count and viability	Low	*In Vivo* *In Vitro*	Mouse (*56*) Rat (*57-59*) Human (*60*) Mouse (*61*)

The most frequently used cytotoxic antibiotic in oncological treatment is doxorubicin. It is an anthracycline antibiotic widely used in the treatment of oncologic diseases such as leukaemia, neuroblastoma, sarcomas and other malignant tumours (*53*). Furthermore, it is commonly implemented in the oncological treatment protocols of pre-pubertal patients (*2, 62*). This drug is a very effective antineoplastic agent due to its aptitude to block DNA and RNA polymerase activity, inhibit topoisomerase II and interfere with chromosomal stability, thus ending DNA and RNA synthesis and blocking the cell cycle (*53, 63, 64*). As a result, several studies have been done to determine its implications on male fertility. Research conducted in mice has shown that doxorubicin is responsible for, in a dose dependent manner, reducing sperm count, motility and morphology (*52*). Furthermore, this drug also affects the structure and function of Sertoli cells and prevents spermatids from entering the spermiogenesis phase (*52*). Similar results were also described for male rats (*54*). Vendramini and collaborators tested the gonadotoxic effects of doxorubicin on male rats, and have observed that this drug is damaging to the seminiferous epithelium. Furthermore, in the follow-up of adult rats treated with doxorubicin, they discovered that this agent is also

responsible for an increase in embryo loss, probably due to genetic abnormalities in spermatogonia (*53*).

Dactinomycin acts as an inhibitor of nucleic acid synthesis, preventing production of DNA and RNA, being also capable of stopping protein synthesis, by interrupting the peptide-chain elongation or preventing the attachment of oligosaccharide side chains to glycoproteins (*65, 66*). Dactinomycin is implemented as a single agent or in combination with other chemotherapeutics in several treatment protocols for children, young adults and adults, being responsible for high cure rates in Wilms tumour, rhabdomyosarcoma and Ewings sarcoma (*67*). Even with a high cure success rate, its effects on fertility must be taken into consideration when prescribing such antibiotic. Dactinomycin is capable of penetrate the blood-testis barrier, directly affecting all germ cells (*34, 68*). Studies in mice have shown that dactinomycin preferentially eliminates spermatogonia A and is responsible for alterations in spermatogenesis that yield deformed round spermatids with a diploid nuclei. As a result, the administration of these agent single-handedly reduced sperm count (*34*). Follow-up of male patients after treatment with combination therapies with dactinomycin revealed an infertility rate of 28% (*55*).

Bleomycin is mainly employed in the treatment of squamous cell and testicular carcinomas, lymphomas, malignant pleural effusions and ascites (*69, 70*). This cytotoxic antibiotc exerts its antineoplastic activity by interacting with nucleic acids and proteins, leading to chromatin cleavage and cellular death (*71*). *In vitro* studies conducted by Liu and collaborators on mice, revealed that this drug is capable of directly damaging germ cells' DNA, including the telomere region (*61*). Data obtained on rats supported the previous findings and revealed that this agent induces genetic and epigenetic mutations in all male germ cell types (*56, 59*) and, as a result of germ cell depletion, alters testis histology (*57*). Though studies have shown that bleomycin is gonadotoxic, its effects are usually temporary and normal spermatogenesis is regained a few months after treatment (*58*). The evaluation of men exposed to chemotherapies with bleomycin presented similar results, with bleomycin causing only temporary azoospermia (*60*).

2.3 Antimetabolites and related therapy

Antimetabolites and related therapy are effective antineoplastic agents, with moderate toxicity, normally used in combination with other chemotherapeutics to achieve a more effective regimen, due to substances synergism (*72*). Antimetabolites are elements that mimic a natural occurring molecule of the cell and interfere with all surrounding physiological pathways (*73*). By interfering with cell metabolism, these drugs are capable of altering cell functions. Antimetabolites that specifically target cell pathways, crucial for tumour growth,

can induce cellular arrest and death (*74*). There are some antimetabolites with antineoplastic activity that are routinely used in the medical practice to treat leukaemia and a number of solid neoplasia (*72, 73, 75*). These agents have several detrimental effects on male fertility (Table I-3).

Table I-3. Antimetabolites and related therapy and their adverse effects on male fertility

Chemotherapeutic drug	Formula	Effect on male fertility	Infertility Risk	Study Type	Species
Cytarabine (Ara-C)	$C_9H_{13}N_3O_5$	Altered testes histology Reduced in sperm count and quality	Moderate	*In Vivo* *In Vitro*	Mouse (*76, 77*) Rat (*78*) Mouse (*77*)
5-Fluorouracil (5-FU)	$C_4H_3FN_2O_2$	Damaged spermatogonia and spermatocytes Reduced sperm count and quality	Moderate	*In Vivo*	Mouse (*34, 79*) Rat (*80, 81*)
6-Mercaptopurine (6-MP)	$C_5H_4N_4S$	Damaged to spermatids Reduced sperm count	Low	*In Vivo*	Mouse (*34, 82*) Human (*83-85*)
Methotrexate (MTX)	$C_{20}H_{22}N_8O_5$	Testicular toxicity Germinal cell line degeneration Reduced sperm count and quality Oligospermia	Moderate	*In Vivo*	Mouse (*34, 86-90*) Rat (*91, 92*) Rabbit (*93*) Human (*94, 95*)

Cytarabine (Ara-C) is one of the most effective agents in the management of acute leukaemia and other hematopoietic malignancies. Its effectiveness as antineoplastic agent is the result of its ability to interfere with DNA polymerases and incorporate into DNA strands (*96*). Hence, Ara-C is capable of inhibiting DNA synthesis, leading to DNA fragmentation and consequently cell apoptosis (*96, 97*). Even though this drug is widely used in the treatment of children with leukaemia, it conveys many side effects. In males, the testes are particular affected. *In vivo* studies performed in male rats revealed that the sole administration of this agent modifies the testes histology and is responsible for a reduction in sperm count and quality. Moreover, Ara-C (50 to 200 mg/kg/day) diminished spermatogonia proliferation, delaying the start of spermatogenesis (*78*). When investigating the effects of Ara-C on male mice, Palo and peers (*76*) confirmed previous results and reported that Ara-C (100, 150 and 200 mg/kg) is liable for high percentages of abnormal spermatogonia and primary spermatocytes in the seminiferous tubules, as well as an elevated numbers of atypical sperm in the epididymis (*77*).

Another antimetabolite commonly used in cancer treatments is 5-fluorouracil (5-FU), an agent from the fluorinated pyrimidines family, specifically designed according to scientific data of tumour biochemistry (*98, 99*). Despite being highly effective and routinely used

against head, neck and gastrointestinal tumours, it is known that this antimetabolite induces chromosomal aberrations and mutations. In addition, it is a potent gonadotoxic that causes testicular damage, halts cell maturation and leads to the formation of deficient spermatids (*81*). In fact, several studies in rats have shown that 5-FU toxic side effects are dose-dependent, causing the shrinkage and atrophy of the seminiferous tubules and altering spermatogenesis eventually leading to a significant decreased in sperm count (*80, 81*). Likewise, results obtained in male mice demonstrated that a single dose administration of 5-FU induced a statistically significant number of DNA mutations in spermatogonia and spermatocytes, eventually killing the latter, in addition to altering sperm morphology. These findings suggest that the mutations induced by this drug in spermatogonia disrupt the complete course of spermatogenesis, ultimately affecting spermatozoa (*34, 79*).

6-mercaptopurine (6-MP) is a purine antagonist generally used in the treatment of pre-pubertal acute lymphoblastic leukaemia (*82, 100*) and in the management of bowl diseases (*84, 85*). The daily administration of this drug in combination with other cytotoxic chemotherapeutics lengthens the period of remission. In fact, 60% of the individuals treated with combination therapies including this drug are in complete remission after a 5 year period (*100*). This antimetabolite interferes with all biological mechanisms involving endogenous purines, crucial constituents of the cell genetic information. Through the formation of its intracellular metabolite, thioinosinic acid ($C_{10}H_{13}N_4O_7PS$), 6-MP leads to the inhibition of DNA, RNA and protein synthesis, exhibiting both cytotoxic and immunosuppressive properties (*101*). The detrimental effects of this chemotherapeutic on male fertility have been assessed in several studies. The administration of 6-MP to male mice has been linked with a general reduction in fertility. This drug compromised spermatids viability, being responsible for a decrease of as much as 25% in sperm count (*34*). Ultimately, its administration to male individuals resulted in a considerably high rate of embryonic reabsorption (*82*). The use of 6-MP in men that wish to become fathers is dubious. There are evidences that, if men are exposed to this agent within 3 months prior to conception, there are higher chances for foetus malformations and gestational complications (*84, 85*).

One other drug specifically developed for the oncological treatment is methotrexate (MTX). It has several pharmacological properties, being not only an effective anti-proliferative agent, but also an immunosuppressor and anti-inflammatory drug. This agent is a folate antagonist regularly used to treat malignancies, such as acute lymphoid leukaemia, osteosarcomas and bladder cancer (*102, 103*). Being a folate antagonist, this element binds to the dihydrofolate reductase enzyme, preventing the synthesis of purines and pyrimidines and consequently disrupting protein synthesis and leading to cell death

(*104, 105*). In males, spermatogenesis may be particularly affected by MTX, causing important fertility issues. Until now several studies have been conducted in many different species and there are quite a few case reports of the side effects of this drug on male fertility. While testing the toxic effects of MTX on male mice germ cells, Choudhury and peers discovered that, after intraperitoneal administration (2, 10 and 20 mg/kg) of this agent there was a significantly higher percentage of abnormal primary spermatocytes and morphologically altered spermatozoa (*86*). There were also some evidences that MTX acts as a clastogenic agent inducing cytogenetic alterations in spermatogonia, which were transmittable to sperm (*86, 88*). Other studies in mice confirmed previous results, allowing the classification of MTX as a clastogenic agent capable of causing DNA damage to male germ cells, reducing sperm count and overall quality and inducing general testicular toxicity (*87, 89, 90*). Results obtained on rats exposed to an intravenous administration (100, 300. 500, 700 mg/Kg) of MTX also revealed that the lower and intermediate dosages tested may possibly lead to oligospermia, but do not cause testicular atrophy (*91*). These findings may be the result of minor changes in testicular histologic parameters, among an accentuated apoptosis of germ cells and compromised spermatogenesis, as demonstrated in rabbits (*93*) and in rats (*92*). Due to its diverse pharmacological properties, there are some case reports on the effects of MTX on human male fertility that may be useful to predict fertility on cancer patients after MTX treatment. In a 1993, a case report described an infertile patient with psoriasis, presumably due to recurrent treatments with MTX (*94*). They reported that the fertility issues arose from genetic abnormalities and subsequent gene mutations triggered by this drug and recommended that male individuals should be counselled before initiating MTX therapy. A more recent report in a patient with severe Crohns's disease had similar results, suggesting that this drug must not be prescribed to men trying to conceive, because of its presumable teratogenic properties, even though this not always seem to be the case and some men have perfectly healthy children (*95*).

2.4 Vinca Alkaloids and other natural products

Throughout human history, natural products have played a critical role in medicine. Plants have been studied for their pharmacological properties since the origin of civilization, but only recently their antineoplastic qualities have been assessed. Nowadays some phytochemicals are the main component of several chemotherapeutic regimens (*106-108*). Plants are a natural source for antineoplastic agents, there are plant produces that affect the overall male reproductive function (Table I-4).

Table I-3. Vinca alkaloids and other natural products and their effects on male fertility

Chemotherapeutic drug	Formula	Effect on male fertility	Infertility Risk	Study Type	Species
Vinblastine (VBL)	$C_{46}H_{58}N_4O_9$	Changes in germ cell populations Induction of chromosomal aberrations	Moderate	*In Vivo*	Mouse (*109, 110*) Human (*111*)
Vincristine (VCR)	$C_{46}H_{56}N_4O_{10}$	Changes of the seminiferous tubules Arrest of spermatogenesis at the spermatid phase Azoospermia	Moderate	*In Vivo*	Rat (*112*) Human (*60, 113-115*)
Etoposide	$C_{29}H_{32}O_{13}$	Temporary gonadal damage Distress of spermatogenesis Increased chromosomal abnormalities in sperm	Moderate	*In Vivo* *In Vitro*	Rat (*116*) Human (*31, 117-120*) Human (*121*)

The first natural chemotherapeutic agents derived from plants to be developed and used in the clinical practice were the vinca alkaloids vinblastine (VBL) and vincristine (VCR). These agents are isolated from the Madagascar periwinkle, *Catharanthus roseus*, and are known to interfere with some biological functions (*108, 122*). Vinca alkaloids are cytotoxic agents capable of binding to tubulin preventing dimer polymerization and therefore interfering with the assembly of microtubules (*109, 123*). Hence, these agents have strong anti-proliferative properties due to general mitotic arrest (*123, 124*).

VBL is a frequently used chemotherapeutic agent in the treatment of several malignancies, like germ cell cancers and Hodgkin's lymphoma (*109*). This drug is capable of arresting the cell division cycle in metaphase, thereby inhibiting cell proliferation. Furthermore, VBL is known to induce aneuploidy and indirectly lead to chromosomal aberrations (*124*). Chemotherapeutic treatments with VBL have been related with the onset of azoospermia, which may be reversible after a period of 3 years in approximately half of the affected men (*109*). As a result, there is general concern on the reproductive and genetic integrity of these men and their progeny after treatment (*109, 110*). A study conducted on mice revealed that VBL is cytotoxic to male reproductive functions at different dosages. While analysing testicular weight, researchers found that, at dose of 1 mg/kg or higher, there was a significant decline in testicular weight. In addition, administration of VBL resulted in significant changes in germ cell populations (*109*). Recent reports on VBL genotoxicity disclosed that this agent is accountable for significant changes in the cell cycle, ultimately leading to an increase in various chromosomal aberrations in mice (*110*). Due to the known capacity of VBL to induce genetic damages that may be transmissible to the

offspring, Robbins and peers studied male patients that underwent treatment for Hodgkin's disease with this drug. They described that in these patients there was an increase in spermatozoa with chromosomal defects (*111*).

VCR is also a highly effective, cell cycle specific, chemotherapeutic agent often used in the treatment of diverse cancer types (*125*). Several studies have found that the VCR efficacy as an anti-proliferative drug rests on its concentration and duration of exposure, as well as on the amount of cancer cells in mitosis at the time of treatment (*126*). Despite its wide use in the clinical practice, VCR has some noticeable secondary side effects on male reproductive functions. A study conducted on male rats revealed that VCR significantly impaired male fertility and was responsible for seminiferous tubules histological changes (*112*). Furthermore, this drug was responsible for the arrest of spermatogenesis at the spermatid phase. Although most VCR-treated male rats recovered fertility several weeks after cessation of treatment, a third did not, and remained permanently sterile (*112*). In humans, VCR is routinely used as part of diverse combination therapies. Therapies containing VCR are usually implemented in patients with Hodgkin and non-Hodgkin lymphoma and are responsible for increased damages on male spermatogenesis (*60*). Rautonen and peers (*115*) studied the effects of VCR on male fertility and concluded that, this agent, when administered in children or young adults, may be the main culprit in causing possibly permanent azoospermia. In fact, therapy containing this agent is accountable for the extreme impact of treatment on human male fertility, which in most cases may be irreparable (*113, 114*).

In addition to the chemotherapeutics from the vinca alkaloid family, there are several others that derive from natural products. Etoposide is a semi-synthetic agent that derives from the podophyllotoxin, extracted from a plant commonly known as Mayapple, *Podophyllum peltatum* (*106, 127*). This drug has long been used as a first-line agent in the treatment of numerous neoplasia, such as lung and testicular cancer, non-Hodgkin's lymphoma, leukaemia and sarcomas (*128*), and was the first antineoplastic drug discovered to act via topoisomerase II inhibition (*129*). Topoisomerase II inhibitors are among the most powerful antineoplastic agents and are therefore used in multiple cancer types (*130*). Etoposide is a phase-specific, cytotoxic drug that acts by stabilizing the topoisomerase II complex with DNA, which directly results in DNA strand breaks and leads to cell death by apoptosis (*131*). Moreover, there are also evidences that etoposide does not only cause DNA breaks by topoisomerase II interaction, but also the formation of free radicals (*132*). Stumpp and peers (*116*) investigated the incidence of apoptosis in the seminiferous epithelium of adult rats treated with etoposide during the pre-pubertal phase. Their results revealed that this drug, when administered during the pre-pubertal phase impaired male

fertility. Etoposide is commonly implemented in the clinical practice as part of a combination therapy. Thus, many authors have followed etoposide treated patients and observed its consequences on male fertility. Ishikawa and peers have analysed the effects of etoposide in spermatogenesis and have described that its recovery after chemotherapy was only observed in some of the patients (*31*). Similar results have been attained by Stephenson and peers (*118*). On the other hand, a similar study found no significant permanent damage in the male gonads (*117*). However, the chemotherapeutic regimen followed was different what may explain these conflicting results. While analysing the genetic damage caused by etoposide in human spermatozoa, De Mas and peers found that there was an increase in the number of aneuploidies, probably due to etoposide mode of action (*120*). However, an analogous study found no increase in human sperm chromosomal abnormalities (*119*). Nonetheless, two times more spermatozoa per patient and chromosomes was analysed by De Mas and peers, what may explain the differing results between the two studies. *In vitro* studies on etoposide are scarce but revealed that this drug is capable of severely distressing human spermatozoa (*121*).

Chemotherapy agents have a broad spectrum of damaging effects on male fertility. However, there is still the need to further study the majority of these agents in order to improve the treatment given to patients. In addition to the agents described in this review, several others may be affecting male fertility but are yet to be identified.

3. Fertility preservation in men undergoing chemotherapy

An ideal cancer treatment would only target malignant cells and would have no secondary effects on the gonads. However, most oncological drugs target mainly dividing cells, which makes them highly detrimental for germ cells and fertility, especially in the male. Even though therapeutic regimens are being constantly improved, with less gonadotoxic approaches, they are still harmful, and their outcome on male fertility is difficult to predict (*5, 15*).

Currently, there are no complementary therapies available that are entirely effective in preventing damages caused by chemotherapeutics to male reproductive functions (*12, 133*). Having this in mind, there is a clear need to discover agents capable of protecting male fertility during the chemotherapeutic regimens or agents that are able to restore normal reproductive functions after treatment completion.

In general, chemotherapeutics affect the patient's semen parameters, like concentration, motility and morphology, the spermatozoa genetic content, the overall testicular histology and the hormonal levels (*31, 119, 134*). The intensity of the damages typically rests on the chemotherapeutic drug and dosage used, as well as the treatment

length. All these factors also influence the reversibility of the damages and ultimately the recovery of fertility (*14, 135*). Antineoplastic agents affect non-cancerous tissues in different manners, so there is the need to find agents that act specifically on those pathways and are able to protect those cells. Hence, there are different strategies for male fertility preservation against the chemotherapy side-effects and protector agents can be assembled into different categories depending on their means of action (Figure I-2).

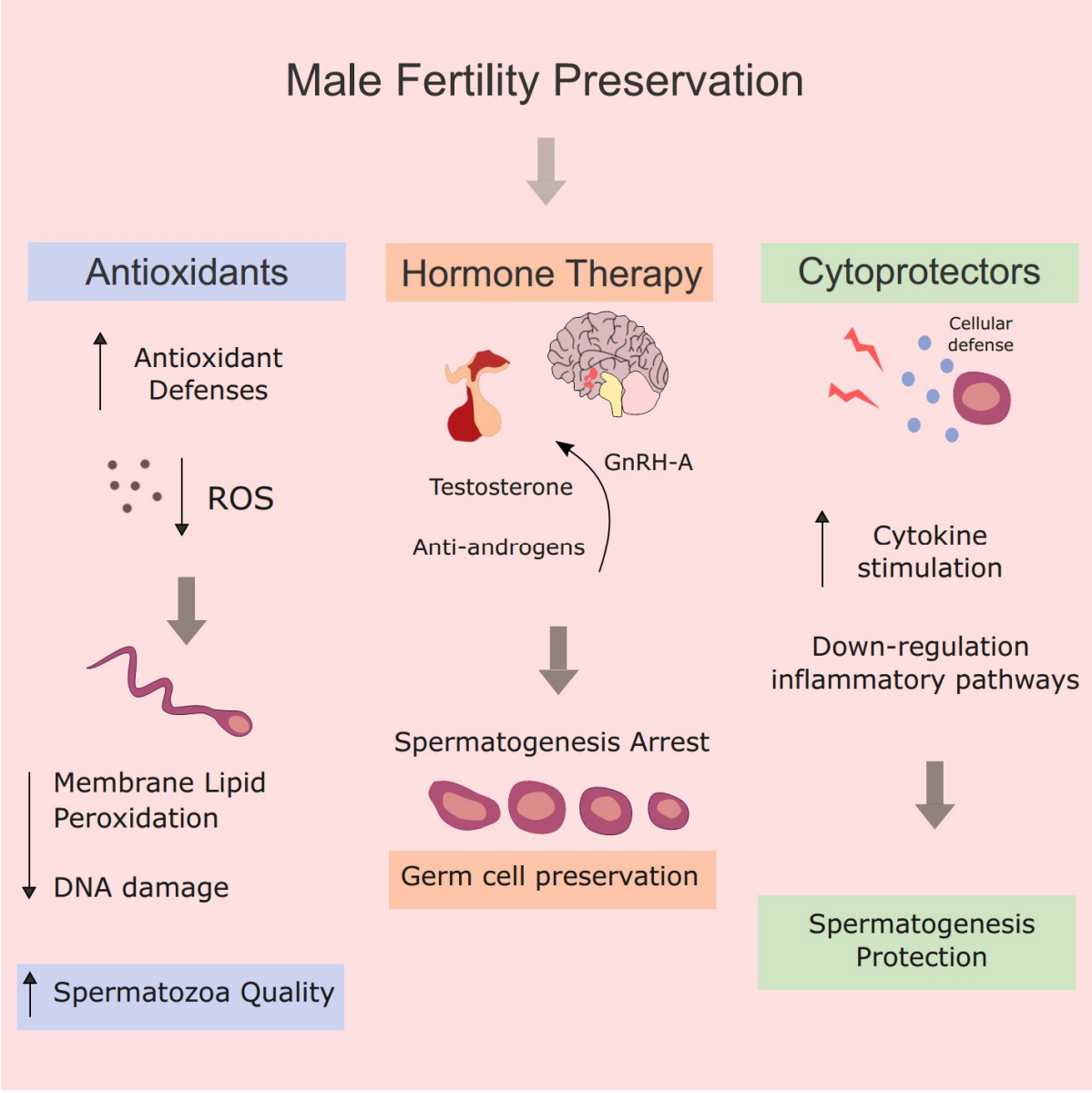

Figure I-2. Fertility preservation strategies for males being treated with cytotoxic chemotherapies. Three preferential paths are being tested as fertility preservation options for males undergoing chemotherapy: antioxidant defence, hormone therapy and the use of cytoprotector agents. These paths focus on improving spermatozoa quality, preserving germ cells and protecting spermatogenesis. Abbreviation: ROS, reactive oxygen species; GnRH-A, gonadotropin-releasing hormone antagonist.

3.1. Antioxidants

Oxidative stress has been identified as one of the primary causes of the gonadotoxicity of anticancer therapies. Reactive oxygen species (ROS) are naturally generated *in vivo* from dioxygen (O_2). Although they are crucial players on several cellular processes, of which cell signalling is one of the most relevant, their derivatives, like hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), can be highly detrimental for tissues and cells (*136, 137*).

There are numerous biological antioxidants and enzymes that are responsible for the preservation of cellular redox conditions. However, when there is an imbalance between ROS production and its elimination, cells enter in oxidative stress. This condition incites several oxidative reactions that ultimately may lead to cellular damage and death (*136, 138*).

More than 40% of all approved antineoplastic agents are known to induce oxidative stress both in cancerous and non-cancerous tissues. In fact, tissue damage due to oxidative stress is one of the most substantial side effects of chemotherapy (*136*). Oxidative stress is harmful to testis and spermatozoa being one of the hypothesised mechanisms responsible for male infertility (*139, 140*). Consequently, there is a growing number of antioxidants being tested in order to identify those with the highest potential in protecting the reproductive function of males undergoing chemotherapy (Table I-5).

Table I-5. Antioxidants with protective effects against chemotherapy secondary effects on male fertility

Antioxidant	Formula	Effects	Efficiency	Study Type	Species Studied
Astaxanthin (AST)	$C_{40}H_{52}O_4$	Improved reproductive parameters Reduced chemotherapy induced DNA damaged	High	*In vivo*	Mouse (*141*)
Lycopene	$C_{40}H_{56}$	Normalized reproductive parameters Improved sperm concentration and motility	Moderate	*In vivo*	Rat (*142-144*)
Ellagic acid (EA)	$C_{14}H_6O_8$	Decreased sperm abnormalities Improved sperm concentration and motility	High	*In vivo*	Rat (*144-147*)
α-Lipoic acid (LA)	$C_8H_{14}O_2S_2$	Normalized reproductive parameters Improved testicular histology	Moderate	*In vivo*	Rat (*148-150*)
1-N,4-N-diphenylbenzene-1,4-diamine (DPPD)	$C_{18}H_{16}N_2$	Improved testicular histology	High	*In vivo*	Rat (*151*)
Melatonin	$C_{13}H_{16}N_2O_2$	Improved testis histology Decreased induced spermatogenesis toxicity Improved the cytotoxic damages found on germ cells	High	*In vivo*	Mouse (*33, 152*) Rat (*153*)
N-acetylcysteine (NAC)	$C_5H_9NO_3S$	Reduced lipid peroxidation Reduced chemotherapy induced oxidative stress	Moderate	*In vivo*	Rat (*151, 154*)
Vitamin C (Ascorbic acid)	$C_6H_8O_6$	Protected male testes Partial reversion of gonadotoxicity Improved fertility recovery	Moderate	*In vivo*	Mouse (*155*) Rat (*151, 156, 157*)

Astaxanthin (AST) is an antioxidant from the carotenoid family. Antioxidants from this category function as important protectors of biological structures against oxidative stress mainly acting as free radical scavengers (*158*). AST is a naturally occurring antioxidant usually found in various crustacean species, algae and yeast. AST is capable of trapping free radical on the outer and inner cellular membranes thereby, effectively inhibiting lipid peroxidation (*159*). These characteristics make AST one of the most powerful antioxidants of its family. In fact, it has been reported that this antioxidant is 10 times stronger than others of its category (*160*). Its powerful antioxidant characteristics make it an ideal candidate to potentially protect cells from the damaging effects of oxidative stress. Taking this into consideration, Tripathi and Jena, studied the protective effects of AST against the

secondary effects of cyclophosphamide on male mice germ cells (*141*). These authors assessed several reproductive features and discovered that, as hypothesised, treatment with AST significantly improved testes histology and prevented the cyclophosphamide-induced damage to spermatozoa, restoring their normal head morphology. Besides, AST was able to significantly reduce the germ cell's DNA damage (*141*).

Lycopene is another important antioxidant from the carotenoid family. This compound has very strong antioxidant properties and can be found at high concentrations in numerous fruits and vegetables, especially in tomato (*161-163*). Due to its structural configuration, lycopene is a potent antioxidant with high singlet-oxygen quenching capability that can reduce several oxidative stress biomarkers. Therefore, this compound can prevent free radicals from disrupting cellular balance, being highly beneficial for general health (*164, 165*). Besides its antioxidant ability, there are many other positive features being investigated and studies have shown that lycopene appears to also have some anti-proliferative and anti-carcinogenic activities (*166, 167*). It has also been tested as a supplemental treatment for human male idiopathic infertility and it has shown some promising results. Lycopene was capable of increasing sperm concentration and movement trough free radical scavenging ultimately improving gestation rates (*168*). Accordingly, several studies have been made on the hypothesis that lycopene can protect male reproductive functions from the oxidative assault of chemotherapy. Atessahin and peers (*142*) suggested that lycopene could protect rat sperm from cisplatin-induced toxicity. In their study, rats were treated with an intraperitoneal dose of 7 mg/kg of cisplatin. To observe the protective effects of lycopene they divided their experiment into two distinct groups. One group of animals was pre-treated with lycopene for ten days prior to cisplatin administration and the other received lycopene for five days after the cisplatin was administrated, both experimental groups received a dose of 4 mg/kg lycopene. The authors discovered that lycopene was able to generally improve the reproductive capacity of the male rats. However, the more significant results were found in the post-treatment group. In fact, rats treated with lycopene after cisplatin administration displayed a significant normalization in the sperm concentration, motility and morphology. In addition, the redox status of spermatozoa was also significantly improved by the post-administration of lycopene, with the regularisation in both malondialdehyde and glutathione levels. These results were later corroborated by Turk and peers (*143, 144*). These authors performed studies to assess lycopene ability to protect rat testes from two distinct drugs, cyclosporine A and cisplatin. They found that the simultaneous administration of lycopene with these drugs is able to successfully preserve the rat spermatozoa and testes histology, thereby preserving its reproductive capacity.

1-N,4-N-diphenylbenzene-1,4-diamine (DPPD) is a potent synthetic antioxidant. This compound is able to donate a hydrogen to a radical derivative thus breaking the autocatalytic cycle (*169*). DPPD acts as a free radical and peroxidation inhibitor (*170*). A study has been conducted on its ability to protect rat testes from the oxidative damage caused by cisplatin. Authors have found that DPPD is able to significantly reduce cisplatin-induced oxidative stress and improve testes histology. When compared with other antioxidants, DPPD proved to be more effective in reducing the number of apoptotic testicular cells (*151*).

Ellagic acid (EA) (2, 3, 7, 8-tetrahydroxy [1]-benzopyrano [5, 4, 3-cde] [1] benzopyran-5, 10-dione) is a naturally occurring polyphenol present in a variety of fruits and vegetables. This compound has several biological activities beneficial for human health, among which its antiviral, anti-inflammatory and antioxidant properties are the most noticeable (*171*). The main quality responsible for this wide range of activities is its antioxidant and free radical scavenger capacity that has been extensively studied (*172-174*). Nonetheless, the exact antioxidant mechanism of action of this compound is still unclear (*147*). EA is constituted of four hydroxyl and two lactone groups, in which the first is known to intensify the antioxidant activity, preventing lipid peroxidation, and protecting cells from the damage induced by oxidative stress (*145*). Besides being on itself a remarkable antioxidant, its metabolites are also being studied for their antioxidant abilities (*174*). This distinctive trait makes EA one of the most interesting compounds to address while studying oxidative stress inhibitors. Several studies have been conducted in order to identify its protective effects on male gonads exposed to chemotherapy. Turk and peers (*144, 145, 147*) conducted numerous studies on the protective effects of EA against the detrimental effects induced to the male rat gonads by different chemotherapeutic drugs, such as cisplatin and cyclosporine. Their results revealed that EA amended the chemotherapy induced damages to testes, epididymis, seminal vesicles and prostate. Furthermore, this antioxidant decreased sperm abnormalities and improved its concentration and motility. The authors concluded that EA antioxidant skills were able to protect male rat reproductive capacity against oxidative stress and toxicity caused by the different chemotherapy regimens. Another study, conducted on male rats by Ceribasi and peers (*146*), evaluated the capacity of EA to protect the male reproductive organs when exposed to chemotherapy. EA provided significant protection to testicular tissue against therapy-related oxidative stress, reducing chemotherapy-damages to male gonads and testicular apoptosis. Nevertheless, authors found that EA was not able to reverse organ weights loss and deteriorated sperm parameters that resulted from chemotherapy administration (*146*).

α-Lipoic acid (LA) is a small molecule with two potential isomers: R-LA and S-LA (*175*). This biological thiol antioxidant is naturally found in mitochondria were it mainly functions as an essential coenzyme in metabolic reactions (*176*). Its main biological functions are intimately associated with its antioxidant, anti-mutagenic and anti-carcinogenic properties (*175, 177*). Once inside the cells, LA is quickly absorbed and transformed by cells were it can regenerate various endogenous antioxidants and be an effective ROS scavenger, with the ability to restore the cellular redox state (*178*). Several studies have been led in order to take advantage of these traits for the protection of male reproduction during chemotherapy. Results obtained on male rats exposed to cytotoxic antibiotics revealed that LA was able to minimize lipid peroxidation and enhance the quantity and quality of spermatogenesis. Furthermore, LA was likewise capable of reversing the morphological changes induced to the testicular architecture (*150*). A different study led by Selvakumar and peers (*148, 149*) on cyclophosphamide gonadotoxicity further elucidated the antioxidant abilities of LA. This study has shown that this compound can protect male rat testes and semen quality from the oxidative stress derived from chemotherapy. However, as so far all research on this compound has been performed on animal models, several studies on human cells and clinical trials must still be conducted before any clinically relevant conclusions can be drawn.

Melatonin (N-acetyl-5-methoxytryptamine) is a biological molecule mainly synthetized in mammals pineal gland. Besides being largely produced in mammalian cells, melatonin has been reported in edible plants and bacteria (*179, 180*). It is one of the strongest endogenous antioxidants, playing a crucial role in immune response and cell signalling (*181, 182*). Its antioxidant properties have been related to an improvement in the male reproductive dysfunctions related to diverse pathological conditions and exposure to toxic agents (*183*). Melatonin's antioxidant activities can be partly explained by its ability to fuel antioxidant enzymes (*184*), thereby inhibiting lipid peroxidation and DNA damage brought on by oxidation (*185*). It has also been reported that this molecule can inhibit cell proliferation and boost tumour cell apoptosis, consequently having an antineoplastic effect (*186*). Melatonin can easily penetrate biological membranes and so it can directly protect the intracellular complexes from ROS-induced damage (*187*). A study using this molecule has reported that, due to its antioxidant influence, the *in vitro* incubation of human spermatozoa with a 2 mM dose can improve the percentage of motile spermatozoa and reduce the number of non-viable cells (*188*). Several other studies have been performed to assess if melatonin's antioxidant properties can in fact shield male fertility from the damages caused by chemotherapy. A study done using male rats treated with cyclophosphamide and cisplatin, revealed that melatonin (10 mg/kg) protects the male gonads from chemotherapy-

induced damage. In this study, melatonin was able to increase glutathione levels and glutathione peroxidase activity. Furthermore, this compound was able to prevent the histopathological changes provoked by the chemotherapeutic regimen (*153*). One other study using male mice treated with cyclophosphamide obtained similar results. Mice were exposed to different doses of melatonin (2.5, 5, 10, and 20 mg/kg) via intraperitoneal injection for five consecutive days prior to a single administration of cyclophosphamide (200 mg/kg). Authors revealed that all melatonin dosages could considerably lessen the induced spermatogenesis toxicity in mice testes, likely due to its antioxidant properties (*152*). Mirhoseini and peers (*33*) obtained similar results while testing melatonin protective effects against busulfan-induced testicular damage on mice. Their results have confirmed that melatonin significantly reversed chemotherapy-induced cytotoxic damages on mice germ cells.

N-acetylcysteine (NAC) is a derivative of the amino acid L-cysteine, which contains a thiol group, and has chemo preventive and antioxidant characteristics. It is an N-acetyl prodrug that acts primarily on the glutamatergic system (*189*). NAC is available either by prescription in intravenous and vaporizer forms or orally as an over-the-counter product (*190*). This compound is currently used as a mucolytic agent for patients with acute and chronic bronchitis, and as an antidote for paracetamol overdose (*191*). There are several other conditions where NAC use is being tested, however, the most promising result come from its chemopreventive features due to its antioxidant activity (*192, 193*). NAC exerts its antioxidant actions essentially by two mechanisms. It is capable of easily penetrating cellular membranes and, once inside the cells, it is a precursor in the biosynthesis of glutathione. When deacetylated it originates L-cysteine, further assisting glutathione synthesis (*191, 192*). In addition to its intracellular actions, NAC is capable of act at the extracellular level where it acts by directly scavenging free radicals (*194*). It has also been demonstrated that NAC has several positive protective effects on cells and studies on its potential toxicity have demonstrated its safety for clinical use (*195, 196*). Although extensive research has been made in the antioxidant protective effects of NAC on ROS-induced side effects (*197-199*), the amount of studies focused on male fertility is scarce. Yang and peers (*154*) evaluated the protective effects of NAC on Sertoli cell stress induced by sodium fluoride and discovered that this element was able to reduce endoplasmic reticulum stress via ROS inhibition, therefore protecting Sertoli cell functions. This compound has also been studied as a part of an antioxidant combination therapy. Ahmed and peers (*151*) evaluated NAC's antioxidant activity against cisplatin-induced testicular oxidative damage in male rats. Their results showed that those antioxidants could significantly reduce lipid peroxidation and re-establish the cellular redox status. This study presented some very interesting

results that confirmed the potential of NAC antioxidant properties on preventing the gonadotoxic effect of chemotherapeutics.

Vitamin C is an essential nutrient found abundantly in citrus fruits and in a diverse number of vegetables. This vitamin is the biologically active form of ascorbic acid, being responsible for the maintenance of connective tissues and bones (*200*). This compound is an important cofactor for a variety of enzymes and interacts with numerous transcription factors, regulating gene expression (*201*). Vitamin C is also an important antioxidant that functions as a potent reducing agent (*202*). Thanks to its properties, vitamin C is capable of reducing a variety of ROS both in intra and extracellular spaces (*203*). Due to the fact that both cancer and chemotherapy negatively affect men reproductive function partly by inducing oxidative stress and taking into account that vitamin C is a potent antioxidant, several studies have been conducted on its protective effects in male fertility (*155-157, 204*). When evaluating the shielding effects of vitamin C on testes of mice subjected to two chemotherapy cycles of cisplatin (1 and 2.5 mg/kg), Narayana and peers (*155*) concluded that it is in fact capable of partially reversing gonadotoxicity. A number of studies have also been conducted on male rats. Kilarkaje and peers (*156*) demonstrated that Vitamin C, in addition to other antioxidants, is able to protect rat testicular and reproductive functions against combination chemotherapy and increase the recovery process. These results are in agreement with those obtained in humans by Ahmed and peers and Das and peers, which likewise reported vitamin C to be beneficial in the maintenance and recovery of fertility of men exposed to different chemotherapeutic regimens (*151, 157*).

In addition to all the elements and biological molecules previously described, there are several foods of natural origin that have strong antioxidant activity and are potential protectors of the chemotherapy-induced damage to male reproductive function.

One natural compound known for its several pharmacological activities is royal jelly (RJ), a honeybee produce secreted from the glands of worker honeybees. This product is a natural source of proteins, sugars, lipids, vitamins and other nutrients (*205, 206*), and is known for its antioxidant and free radical scavenging abilities. Silici and peers (*205*) evaluated the potential protective effects of RJ against cisplatin-induced toxicity on rat sperm and presented some encouraging results. Authors reported that RJ could prevent the damaging effects to rat sperm brought on by cisplatin, via its antioxidant abilities.

Coffee, and its key component caffeine, are another well-known natural produces with strong antioxidant properties that have been studied as a protector agent against oxidative stress on male fertility. This extremely popular beverage is one of the most consumed in the world and constitutes an important source for bioactive components with antioxidant features (*207, 208*). It has been shown that the antioxidant properties of this product are

important for human health and provide protection against oxidative stress in several organs (*207, 209*). Furthermore, caffeine can significantly reduce protein oxidation and lipid peroxidation in human Sertoli cells (*210*). Consequently, several studies have been performed in order to evaluate its free radical scavenging and anti-peroxidation activity as well as its capacity to protect cells from oxidative stress. A study conducted on male mice exposed to the chemotherapeutic cisplatin, revealed that caffeine could indeed protect testes from the damage provoked by this agent. The results obtained in this study led to the suggestion that caffeine may have an important role in the recovery process of male fertility after the oxidative damage prompted by chemotherapeutic agents (*211*).

In addition to coffee, caffeine is also a major component of teas. Although tea has approximately 1.4 to 3.4 times less caffeine than coffee, there are several countries where tea is more appreciated and consumed and thereby the total amount of caffeine ingested is similar (*210, 212*). Tea is the second most consumed beverage in the world (*213*), and as a result several studies have been conducted on its antioxidant properties and beneficial effects for human health. Its positive effects on male reproduction have been assessed, and so far results have been encouraging (*214, 215*). In brief, the antioxidant capacities of tea must be taken into consideration for the protection of male fertility against oxidative stress induced, for example, by chemotherapy treatment.

3.2 Hormone Therapy

In general, chemotherapeutic drugs target rapidly dividing cells, what suggests that actively dividing germ cells are more susceptible to chemotherapy secondary effects than those of pre-pubertal patients (*216*). In fact, while analysing post-treatment patient recovery it is noticeable that children seem to regain gonadal function and fertility better than adults (*217*). Taking this into consideration, it has been hypothesised that by inducing a quiescent state to the testes with hormone therapy, germ cells would be less susceptible to the damaging effects of cancer treatment and therefore, male fertility would be preserved (*2, 218*).

Studies led on male rats by Pogach and peers (*219*) revealed that a pre-treatment combination of gonadotropin releasing hormone antagonist (GnRH-A) and testosterone is capable of preventing germinal aplasia induced by the chemotherapy. Furthermore, Glode and peers (*220*) have shown that the suppression of gonadal function in male rats by hormone therapy has the potential to protect germ cells of the damaging effects of procarbazine. Moreover, Meistrich and peers and Kurdoglu and peers pre-treated male rats with GnRH-A and anti-androgen flutamide, describing that it could significantly protect spermatogonia from chemotherapy side effects (*221, 222*).

Despite the promising results of animal studies, clinical trials have not been as convincing. In a human clinical it was reported that testosterone administration prior and during chemotherapy could potentially preserve fertility in men (*216*). These results were later seconded by research carried out by Cigni and peers (*217*). They treated men receiving a chemotherapeutic dose of cyclophosphamide with testosterone. Results showed that, although all men became azoospermic or oligospermic during treatment, the great majority regained a normal sperm count and follicle-stimulating hormone (FSH) levels after a twelve month period. Nonetheless, numerous other studies have found hormone therapy to have no protective effects on male gonads exposed to chemotherapy (*223-225*).

3.3 Cytoprotector Agents

Although chemotherapeutic agents are designed to attack neoplastic cells, the truth is that they are highly detrimental for various cell types, including normal cells. Therefore, there is a need to protect normal cells from these damaging effects.

Trichloro(dioxoethylene-O,O') tellurite (AS101), an immunomodulator, is able to stimulate the production of numerous cytokines both *in vitro* and *in vivo (226)*. Many of these cytokines have been described as protectors of the damaging effects induced by chemotherapeutic drugs. Therefore, AS101 is a competent cellular protector with diverse therapeutic applications (*227, 228*). This characteristic of AS101 has been the basis for suggesting that it may be capable of protecting the male reproductive cells from chemotherapy aggressions (*229*). Carmely and peers evaluated the effects of AS101 in the testes of male mice exposed to a dose of 200 mg/kg of cyclophosphamide (*230*). Their results showed that AS101 can in fact significantly protect male reproductive functions against chemotherapy-induced damage by significantly reducing the percentage of damaged seminiferous tubules and reducing sperm DNA fragmentation.

Amifostine ($C_5H_{15}N_2O_3PS$) is an inorganic thiophosphate used in some treatment protocols with the aim of improving patient's life quality. This agent can potentially protect cells by preventing the up-regulation of several inflammatory pathways induced by chemotherapy (*231*). Amifostine recognises cells' physiological environment and can act preferentially on those with an abnormal expression of cellular metabolites (*232*). For that reason, amifostine can specifically protect normal cells, not interfering with the chemotherapeutic treatment. It has been hypothesized that this agent might be able to protect male fertility from the detrimental effects of chemotherapy. Lirdi and peers evaluated amifostine protective action against the damages induced by cisplatin on the testes of male rats (*233*). The results obtained confirmed that its administration before the chemotherapeutic treatment could substantially reduce testicular damage, partly by

reducing the number of apoptotic germ cells. Still, other studies described opposing outcomes. Vendramini and peers (*234*) evaluated the effects of amifostine against doxorubicin therapy in male rats and described that although the seminiferous epithelium damage was reduced, there was no particular improvement in the overall fertility status.

4. Conclusion

Cancer treatment has been improving over the last decades, pushing survival rates to their highest percentage ever. This brings the issue of patient's life quality in the long run to the foreground. A focal point for the overall quality of life of patients is fertility. Fertility is highly important to the great majority of patients (*235*) and must be properly addressed before the gonadotoxic treatment is initiated. Chemotherapy side effects on male fertility are varied and is difficult to assess the individual risk for long term infertility. As a result, several fertility preservation options have been explored and must be discussed with patients.

In order to achieve the best results possible in neoplastic treatment, the therapeutic regimens have been evolving with more effective drugs, generally used in combination. These regimens are more effective in the treatment of malignancies and tend to be less detrimental to male fertility. However, there are still a considerable number of side effects to have into consideration. To achieve a full recovery of sperm production after a cytotoxic insult, stem spermatogonia must be intact and resume their mitotic activity. However, several chemotherapeutic drugs deeply affect spermatogonia, permanently damaging its functioning. The complexity surrounding this issue led to the development of several preservation techniques, and to the exploration of a variety of compounds that could potentially protect male reproductive functions from chemotherapy-induced damage.

Although results on protector agents seem to be promising, there is no certainties on their broad efficiency against chemotherapeutics. There is still the need for a wider research on each protector agent and its full activities on male germ cells when administered together with the various chemotherapeutic drugs. Therefore, in order to preserve the possibility for a future offspring, sperm cryopreservation must be offered before the initiation of treatment.

In the future, further investigation on strategies for preserving male fertility must be pursued. One vital field of research is the discovery of new protector agents, and the investigation of combination supplemental therapies to address the multiple secondary effects brought on by chemotherapeutic regimens. Furthermore, all protector agents must be tested in extended clinical trials before definite conclusions can be drawn and they can be implemented as routine supplemental treatment.

II. Aims

Aims

Chemotherapeutic drugs have a broad spectrum of secondary effects that can severely affect patients' life quality. Even though etoposide is widely used in the clinical practice, the full range of its side effects has not yet been assessed, and studies on its consequences on male fertility are scarce. Consequently the first goal of our project was to analyse etoposide's effects on human sperm parameters, sperm DNA, oxidative stress and metabolism after exposure *in vitro*.

Citoprotection from chemotherapy seems the most reliable option for male fertility preservation so far. As a result we selected an antioxidant, NAC, as a possible protector agent for cells exposed to etoposide due to its clinical safe use and protective properties. Therefore, we aimed to evaluate NAC's ability to preserve human spermatozoa from the damages induced by etoposide.

For these purposes, human sperm samples were obtained and incubated for 2 h alone or in combination with our chemotherapeutic and antioxidant of choice. Microscopic sperm parameters, such as motility, morphology, vitality and viability were measured before and after the incubation period. The damages on sperm DNA were assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and acidic aniline blue (AB) staining. Oxidative stress was evaluated by analysis of carbonyl groups, lipid peroxidation and 3-nitrotyrosine by the Slot-Blot technique. Protein carbonyl content was measured as a marker for protein oxidation, aldehyde products were indicators of lipid peroxidation and nitrotyrosines were a control for protein nitration. Lastly, spermatozoa metabolism was studied by Proton Nuclear Magnetic Resonance (^{1}H-NMR).

III. Results and Discussion

Article II: Chemotherapeutics and male fertility: N-Acetylscysteine Protects Human Sperm DNA integrity during Etoposide exposure

Article II:

Chemotherapeutics and male fertility: N-Acetylscysteine Protects Human Sperm DNA integrity during Etoposide exposure

A. Rabaça[a,b,c], A. Gonçalves[d], Carolina. Ferreira[a,b,e], A. Barros[d,f,g], M.G. Alves[h], M. Sousa[a,b,d], P.F. Oliveira[a,b], R. Sá[a,b,*]

[a] *Department of Microscopy, Laboratory of Cell Biology, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, 4050-313 Porto, Portugal;* [b] *Unit for Multidisciplinary Research in Biomedicine (UMIB), University of Porto, 4050-313 Porto, Portugal;* [c] *Faculty of Medicine, University of Porto (FMUP), 4200-319 Porto, Portugal;* [d] *Centre for Reproductive Genetics Prof. Alberto Barros (CGR), 4100-009 Porto, Portugal;* [e] *University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal;* [f] *Department of Genetics, Faculty of Medicine, University of Porto, 4200–319 Porto, Portugal;* [g] *Institute for Innovation and Health Research (I3S), University of Porto, Portugal;* [h] *Health Sciences Research Centre (CICS), University of Beira Interior (UBI), 6201-506 Covilhã, Portugal.*

Abstract: Etoposide is a chemotherapeutic used in the treatment of several neoplasias that affect males in reproductive age. Fertility recovery after etoposide exposure is associated with the dose used and treatment length, being difficult to predict. Therefore, there is a strong need to identify drugs able to preserve male fertility during etoposide exposure. N-acetylcysteine (NAC), a L-cysteine precursor, has chemopreventive and antioxidant properties. Others have shown that it improves semen parameters and protects Sertoli cells from oxidative stress-induced damages. Since NAC has been reported to possess cytoprotector properties, we hypothesized that it may be a good preserver of sperm quality during in vitro etoposide exposure. In our experiments, human sperm were incubated with 25 µg/ml of etoposide, 50 µM of NAC and both drugs in combination. Sperm parameters as well as DNA fragmentation and chromatin condensation were evaluated. Oxidative damages were assessed and sperm metabolism was studied by proton nuclear magnetic resonance spectroscopy (1H-NMR). Our results demonstrate that in vitro exposure to etoposide induced chromatin alterations and DNA fragmentation. Furthermore, etoposide did not induce sperm oxidative damages nor glycolytic profile alterations. The addition of NAC to sperm exposed to etoposide preserved sperm chromatin condensation and reduced DNA fragmentation. These results suggest that NAC can preserve sperm DNA integrity during etoposide treatment. Therefore, ensuring that for cancer patients who were not advised to cryopreserve sperm prior to chemotherapy treatment, will have sperm DNA integrity preserved and that sperm may be safely used in future Medically Assisted Procreation treatments.

Keywords: Chemotherapy; Etoposide; Male fertility; Spermatozoa; Antioxidant; NAC.

Graphical Abstract:

Protective effects of NAC to DNA integrity of etoposide-exposed sperm.

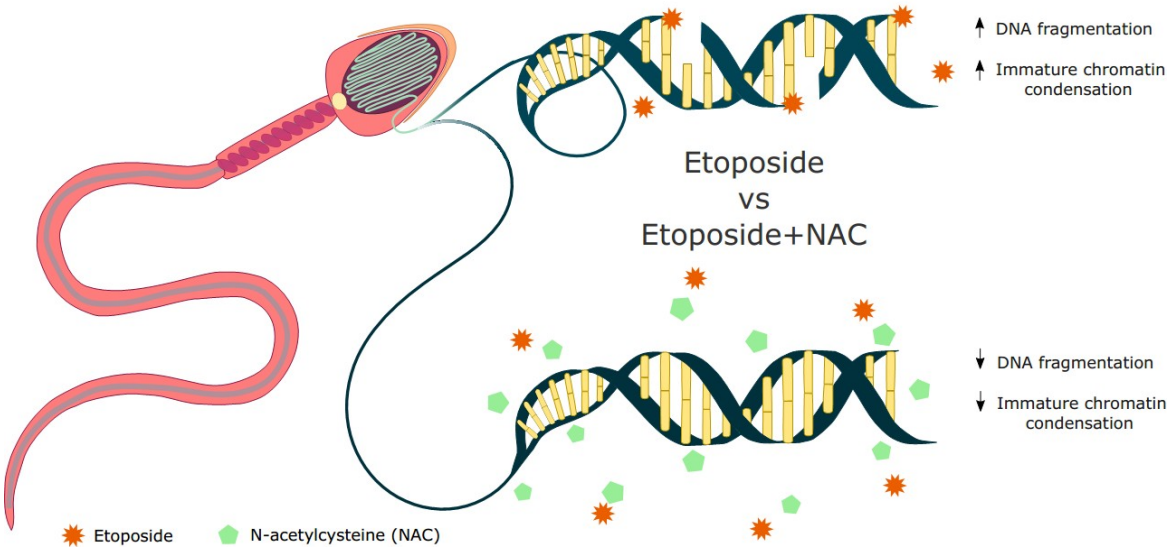

Figure III-1. Protective effects of NAC on sperm DNA integrity.

1. Introduction

Chemotherapeutic agents may interfere with spermatogenesis and cause long-lasting infertility problems (*236*). Etoposide is a semi-synthetic agent that derives from the podophyllotoxin (*132*), widely used, alone or in combination, for the treatment of various neoplasias that affect males in reproductive age (*237-239*). It is mitotic phase-specific and it was the first antineoplastic drug to be discovered to act via topoisomerase II inhibition (*129*). This drug forms a stable complex between topoisomerase II and DNA, highly increasing the number of covalent DNA cleavage complexes, which results in permanent double-stranded breaks (*131*). Besides its interaction with topoisomerase II, etoposide may also cause DNA damage by oxidative stress induction (*132*). Etoposide is known to affect spermatogenesis and thus male fertility (*240*). Studies have shown that etoposide can severely affect spermatogenesis, leading to oligospermia and azoospermia, with only half of the patients regaining reproductive function (*31, 118*). Furthermore, etoposide can damage DNA synthesis being responsible for an increase of aneuploidies in human spermatozoa (*120*). *In vitro* studies on etoposide are scarce, only revealing that its interaction with topoisomerase II is responsible for the distress of human spermatozoa (*121*). Chemotherapeutic regimens are being constantly improved to attain maximum results with the least secondary effects possible. However, most chemotherapeutic regimens are still harmful for male fertility (*15*). Therefore, cytoprotection is an alternative to preserve male reproductive capacity during chemotherapy (*241*). N-acetylcysteine (NAC) is a precursor of L-cysteine presenting chemopreventive and antioxidant characteristics (*189*). It is currently being tested for the treatment of several health conditions. The most promising results come from its chemopreventive features and antioxidant activity (*192, 193*). Studies on its benefits and potential toxicity have demonstrated that this agent is safe for clinical use and has several protective effects on cells (*195, 196*). Furthermore, it has been demonstrated that NAC improves human semen parameters (*242*) and protects rat Sertoli cells from damage induced by oxidative stress (*154*) due to its antioxidant potential.

In the present study, we assessed the toxic effects of etoposide on human sperm and evaluated NAC's potential as chemoprotecting agent of sperm quality during *in vitro* etoposide exposure. Hence, semen parameters, such as motility, viability and morphology, as well as DNA fragmentation and chromatin condensation of sperm exposed to a single dose of 25 µg/ml etoposide alone or in combination with 50 µM of NAC were evaluated. Furthermore, sperm glycolytic and oxidative profiles were also determined.

2. Material and Methods

2.1 Chemicals

Sperm preparation medium (SPM) was purchased from Medicult Origio (Jyllinge, Denmarck). Vectashield antifade medium containing 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA, USA). Protease inhibitor cocktail was purchased from Thermo Scientific (Rockford, USA). Etoposide, NAC, sodium orthovanadate, Triton X-100, Tris buffer, rabbit anti-DNP and rabbit anti-goat IgG-AP were purchased from Sigma (St. Louis, USA). Rabbit nitro-tyrosine antibody was purchased from Cell Signaling Tecnology (Massachusetts, USA). Secondary antibody, goat anti-rabbit IgG-AP was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All the other chemicals were acquired from Merck (Darmstadt, Germany), unless stated otherwise.

2.2 Patients selection and semen collection

Ethical guidelines were followed in the conduct of research, with written informed consent having been obtained before the beginning of work. This work did not involve human or animal experiments. An approval by an Ethics Committee and the Declaration of Helsinki as revised in Tokyo 2004 on human experimentation does not apply to this kind of work. Thus, according to the standards of National Law (PMA, Law 32/2006) and Council (CNPMA, 2008) criteria on Medically Assisted Procreation, 10 semen samples were used after patients had given informed and written consent. Human semen samples were obtained from patients performing spermiogram analysis at the private clinic Centro de Genética da Reprodução Prof. Alberto Barros (CGR-ABarros). The selected group presented a mean age of 33.8±1.2 years. Semen samples were collected by masturbation in sterile containers, after a 3 to 5 day period of sexual abstinence. Semen parameters were evaluated according to the World Health Organization (WHO) guidelines (*243*). Criteria for inclusion were: absence of known pathologies and intake of medicines; normal physical examination, hormonal profiles and karyotypes; semen analysis without agglutination, immature forms, leukocytes and microorganisms, and a sperm concentration ≥ $15x10^6$/ml.

2.3 Experimental design

Semen samples were divided into four groups with different experimental conditions. In the control group (CT), samples were incubated with sperm preparation medium (SPM)

for a 2h period, in a humidified atmosphere of air containing 5% CO2 at 37ºC. The remaining three groups consisted of a 2h incubation period with 25 µg/ml of etoposide (Eto group) or 50 µM of NAC (NAC group), or the combination of both agents (Eto+NAC group).

2.4 Determination of sperm chromatin condensation

Chromatin condensation was assessed by acidic aniline blue staining. Sperm smears were prepared for each condition and fixed in 3% glutaraldehyde in 0.2M phosphate-buffered saline (PBS) solution for 30 min at RT. The slides were then stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH = 3.5) for 5 min at RT. Two hundred spermatozoa were evaluated and the percentage of dark blue stained sperm heads, which indicates immature histone-rich nuclei was calculated.

2.5 Determination of sperm DNA fragmentation

For each sample sperm DNA fragmentation was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany), as previously reported (*244*). Sperm were smeared onto adhesion microscope slides, air-dried and fixed for 1h at RT with 4 % paraformaldehyde in PBS. Slides were then washed in PBS and permeabilized with a solution of 0,1 % Triton-X in 0,1 % sodium citrate for 2min at 4 °C. After washing the slides in PBS, they were incubated in a dark moist chamber for 1 h at 37 °C with a 50 µLTUNEL mixture. After incubation, slides were washed in PBS and counterstained with Vectashield antifade medium containing DAPI. On each slide, at least 200 morphologically normal spermatozoa, without any anomalies in the head, mid-piece or tail, were counted in a Leitz DMRBE fluorescence microscope (Leica, Wetzlar, Germany). The number of spermatozoa emitting green fluorescence (TUNEL-positive) was recorded as a percentage of the total counted sperm (DAPI stained).

2.6 Determination of oxidative stress markers

Oxidative stress can be assessed by: cellular protein carbonyl content, a marker for protein oxidation; measurement of aldehyde products such as 4-hydroxynonenal (4-HNE), an indicator of lipid peroxidation; and quantification of 3-nitrotyrosine (3-NT), an indicator of superoxide-dependent peroxynitrite formation. The content of protein carbonyl groups, 4-

HNE and 3-NT in sperm from the different experimental groups was evaluated using the Slot-Blot technique and specific antibodies, as previously reported (*210*). In brief, total proteins were isolated from human spermatozoa using 1% Triton X-100 in 2M Tris-Buffer, supplemented with 1% protease inhibitor cocktail and 100 mM sodium orthovanadate (phosphatase inhibitor). For carbonyl groups evaluation, protein samples were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain 2,4- dinitrophenyl (DNP) according to the method previously described by Levine and collaborators (*245*). The Slot-Blot technique was performed using a Hybrid-lot manifold system (Biometra, Göttingen, Germany) and the resulting PVDF membranes were incubated overnight at 4ºC with a rabbit anti-DNP (1:5000). For lipid peroxidation and protein nitration analysis, protein samples were diluted in PBS to a concentration of 3 µg/µL. The resulting membranes were incubated overnight at 4ºC with a goat anti-4-HNE antibody (1:5000) and a rabbit nitro-tyrosine antibody (1:5000), respectively. Samples were visualized using rabbit antigoat IgG-AP (1:5000) or goat anti-rabbit IgG-AP (1:5000), respectively. Membranes were then reacted with ECFTM substrate (GE Healthcare, Buckinghamshire, UK) and read using a Bio-Rad Gel Doc XR+ (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using the Quantity One Software Version 4.6.9 (Bio-Rad, Hemel Hempstead, UK).

2.7 Proton Nuclear Magnetic Resonance (^{1}H-NMR)

The extracellular metabolites from each sample after the incubation period were acquired by ^{1}H-NMR, as previously described (*246*). Sodium fumarate at a final concentration of 1 mM was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): H1-α glucose (doublet, 5.22); choline (singlet, 3.18); pyruvate (singlet, 2.38); acetate (singlet, 1.9) and lactate (doublet, 1.33 ppm). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn NMR, Livermore, CA, USA).

2.8 Statistical Analysis

The statistical significance among the experimental groups was assessed by ANOVA, followed by Fisher's LSD. All data are shown as mean ± standard error of mean (SEM) (n = 10 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). All *P values* < 0.05 were considered statistically significant.

3. Results

3.1. Patients selection and sperm examination at collection time

All patients selected for this study were in reproductive age (33.8 ± 1.2 years) and presented normal semen pH values. At macroscopic evaluation, the samples presented normal colour and smell, liquefied in due time and had regular viscosity. Additionally, no leucocytes were found and there was no evidence of agglutination. Furthermore, patients had a sample mean volume of 3.8±0.3 ml, a mean number of sperm per ml of 92.2±21.5 million; a mean percentage of progressive sperm of 46.3±4.9 % and immotile sperm of 31.1±2.9 %, a teratozoospemia mean index of 1.6± 0.0 %, mean sperm vitality of 74.9± 1.8 % and mean hypo-osmolarity of 70.3±2.7 % (Table III-1). No significant differences between patients were found regarding semen parameters at collection time.

Table III-1. Patients' data and semen parameters after sample collection.

Sample Features		Mean	SEM	*P value* [(a)]
Age (years)		33.8	1.19	0.922
Volume (ml)		3.80	0.306	0.338
Nº Spermatozoa / ml		92.2	21.5	0.064
Motility (%)	**Immotile**	31.1	2.86	0.137
	Non-Progressive	22.6	2.93	0.135
	Progressive	46.3	4.89	0.727
Normal morphology (%)		4.75	0.92	0.804
Teratozoospermia index (%)		1.63	0.0374	0.425
Vitality (%)		74.9	1.79	0.268
Hypo osmotic Swelling Test (%)		70.3	2.65	0.303

(a) *P value* within each parameter between patients. *P value* < 0.05 was considered significant.

3.2. Effects on sperm motility

Sperm motility is directly related to male fertility potential and is commonly evaluated to assess male's fertilization capacity [27, 28]. Our results showed that the mean percentage of progressive motile sperm in the control group was 18.6±3.8 % (Figure III-2A). Similar values were found in sperm after exposure to etoposide (19.6±4.5 %). Interestingly, incubation of sperm with NAC, alone or in combination with etoposide, did not significantly alter sperm progressive motility though it was 22.4±5.9 % and 22.7±6.0 %, respectively. Our results also showed that non-progressive motility was not altered in any of the groups in comparison to the control group (data not shown). Likewise our results revealed that the mean percentage of immotile sperm, which was 59.6±6.5 % in the control group, was not significantly altered by exposure to etoposide (61.6±5.6 %), NAC (59.2± 6.8 %) and the combination of both agents (60.3±7.5 %) (Figure III-2B).

3.3. Effects on sperm morphology

It is well-established an association between the percentage of morphologically normal sperm and pregnancy rates [29, 30]. Therefore, sperm morphology evaluation is useful for the assessment of male fertility potential. Our results showed that the mean percentage of normal sperm was 4.8±0.9 % in the control group and was not significantly altered after exposure to etoposide (4.8±0.9 %), NAC (4.7±0.9 %) and the combination of both agents (4.7±1.0 %) (Figure III-2C). Our results also showed that the mean percentage of sperm with head defects in the control group was 94.2±1.1 %. This percentage was not altered in sperm incubated with etoposide (95.5±3.3 %), NAC (95.9±2.3 %) or when both agents were in combination (94.0±3.1 %) (Figure III-2C). No other significant alterations in specific defects, including mid-piece, tail and cytoplasmic excess, were observed in human sperm after incubation with etoposide or/and NAC.

3.4. Effects on sperm membrane integrity

Sperm viability is clinically important since only viable sperm are able to successfully fertilize the oocyte. It can be evaluated by assessing sperm flagellum integrity by the hypo-osmotic swelling (HOS) test [31]. Reactive sperm to the HOS test represent viable sperm with intact membranes. Our results showed that the mean percentage of reactive sperm in the control group was 47.1±2.7 % and was not significantly altered after exposure to etoposide (52.4±3.4 %), NAC (46.3±2.7 %) and the combination of both agents (45.7±3.8

%) (Figure III-2D). Interestingly, the exposure to NAC and the combination of both drugs reduced sperm viability in relation to etoposide (p=0.04 and p=0.03, respectively) but not to controls (Figure III-2D).

3.5. Effects on sperm chromatin condensation and DNA fragmentation

Sperm chromatin is a highly organized and condensed structure, with no distinguishable chromosomes visible. These characteristics protect sperm genetic integrity and facilitate oocyte fertilization [32]. Our results showed that the mean percentage of sperm with immature chromatin condensation was 17.2±1.2 % in the control group. Noticeably, exposure to etoposide significantly increased the percentage of sperm with immature chromatin condensation (26.6±2.8 %) in relation to the control (p=0.02) (Figure III-2E). Sperm treated with NAC (17.5±3.1 %) or Eto+NAC (15.8±1.9 %) presented values similar to the control group, but significantly lower than etoposide group (p=0.04 and p<0.0001, respectively) (Figure III-2E).

Sperm DNA fragmentation is clinically relevant to assess male reproductive capacity [33]. Our results showed that the mean percentage of sperm with fragmented DNA in the control group was 10.8±2.1 % (Figure III-2F). However, after exposure to etoposide, sperm DNA fragmentation highly increased (15.9±0.7 %) in relation to control group (p=0.019). Sperm treated with NAC (10.3±1.6 %) or Eto+NAC (10.6±1.6 %) presented values similar to the control group, but significantly lower than etoposido group (p=0.02 and p=0.02, respectively) (Figure III-2F).

Notably, the addition of NAC to etoposide-exposed sperm was able to protect sperm DNA integrity as results were close to non-exposed sperm.

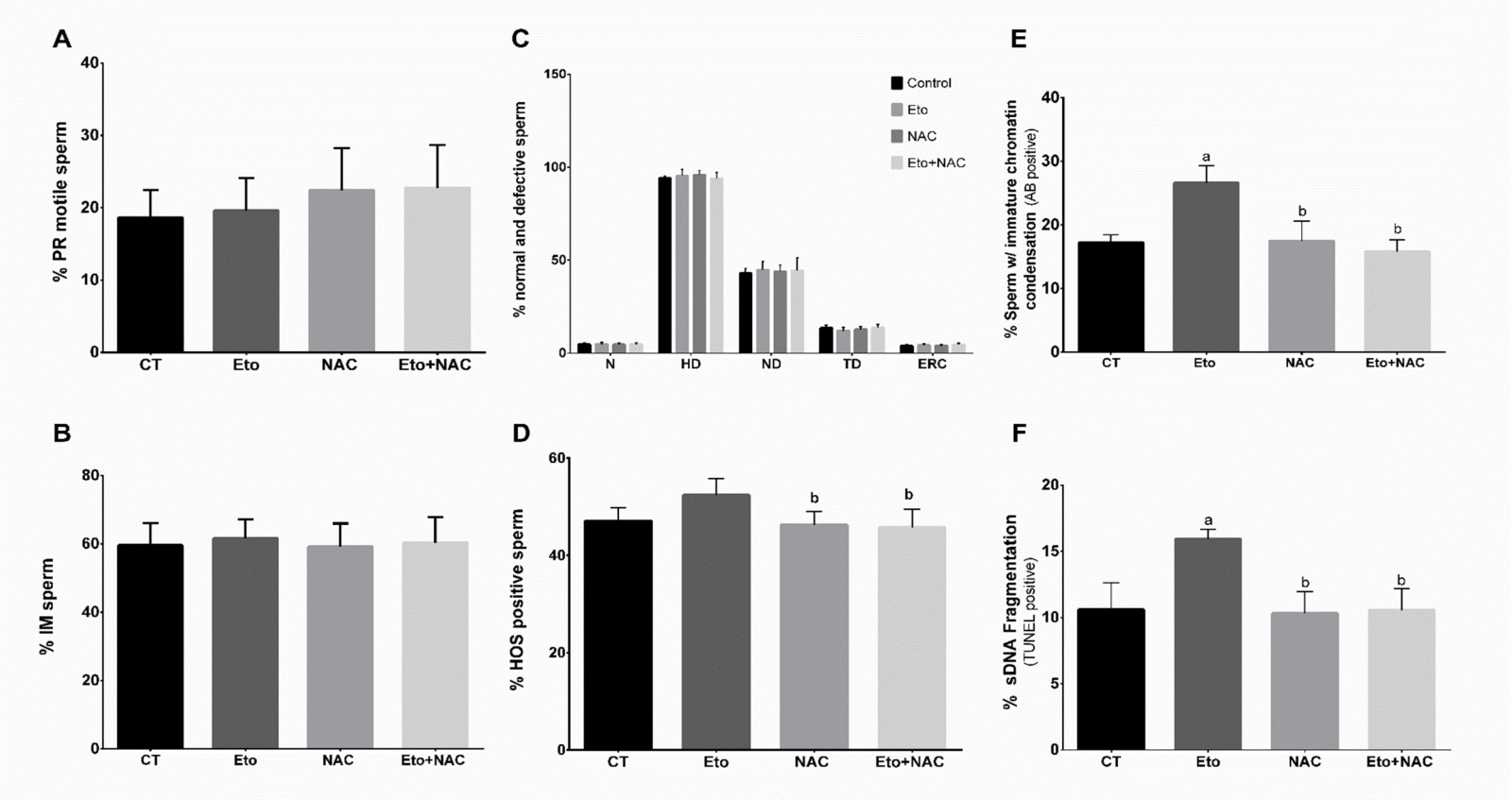

Figure III-2. Effect of etoposide or/and NAC exposure on sperm parameters and DNA integrity. Percentage of sperm with progressive (PR) motility (panel A) and of immotile (IM) sperm (panel B); sperm morphology (panel C), evidencing normal sperm (N), head defects (HD), mid-piece defects (ND), tail defects (PD) and excess of residual cytoplasm (ERC); sperm viability (panel D) assessed by hypo-osmotic test (HOS), immature chromatin condensation (panel E) and DNA fragmentation (panel F) measured after exposure to 25 µg/ml of etoposide (Eto), 50 µM of NAC (NAC) and both drugs in combination (Eto+NAC). Results are expressed as mean ± SEM (n=10 for each condition). Statistical significance was assessed by RM one-way (panel A and B) and two-way (panels C, D, E and F) ANOVA, both followed by Fisher's LSD. Results are expressed as mean ± SEM (n=10 for each condition).Significantly different results ($p < 0.05$) are indicated as: a, relative to control (CT); b, relative to Eto.

3.6. Effects on sperm oxidative stress

Oxidative stress plays a key role in male fertility, being a possible cause for defective sperm, decreased sperm motility and loss of reproductive potential (*247, 248*). Protein carbonyl and nitration, as well as lipid peroxidation, are commonly used markers to evaluate oxidative stress damage. While evaluating protein nitration it was observed that interestingly, the exposure of human sperm to etoposide did not increase nitration levels, presenting values close to those of the control group (1.0±0.1 fold variation to control) (Figure III-3A). However, sperm incubated with NAC or both Eto+NAC displayed significantly higher levels of nitration than those of sperm exposed to etoposide alone [1.1±0.1 fold variation to control ($p=0.04$) and 1.2±0.1 fold variation to control ($p=0.02$), respectively] (Figure III-3A). Furthermore, our results showed that carbonyl groups content was similar in all groups in relation to the control and no significant differences were observed (Figure III-3B). Similarly, the lipid peroxidation levels detected in sperm after incubation with etoposide or/and NAC were close to those detected in the control and no significant differences were noted (Figure III-3C).

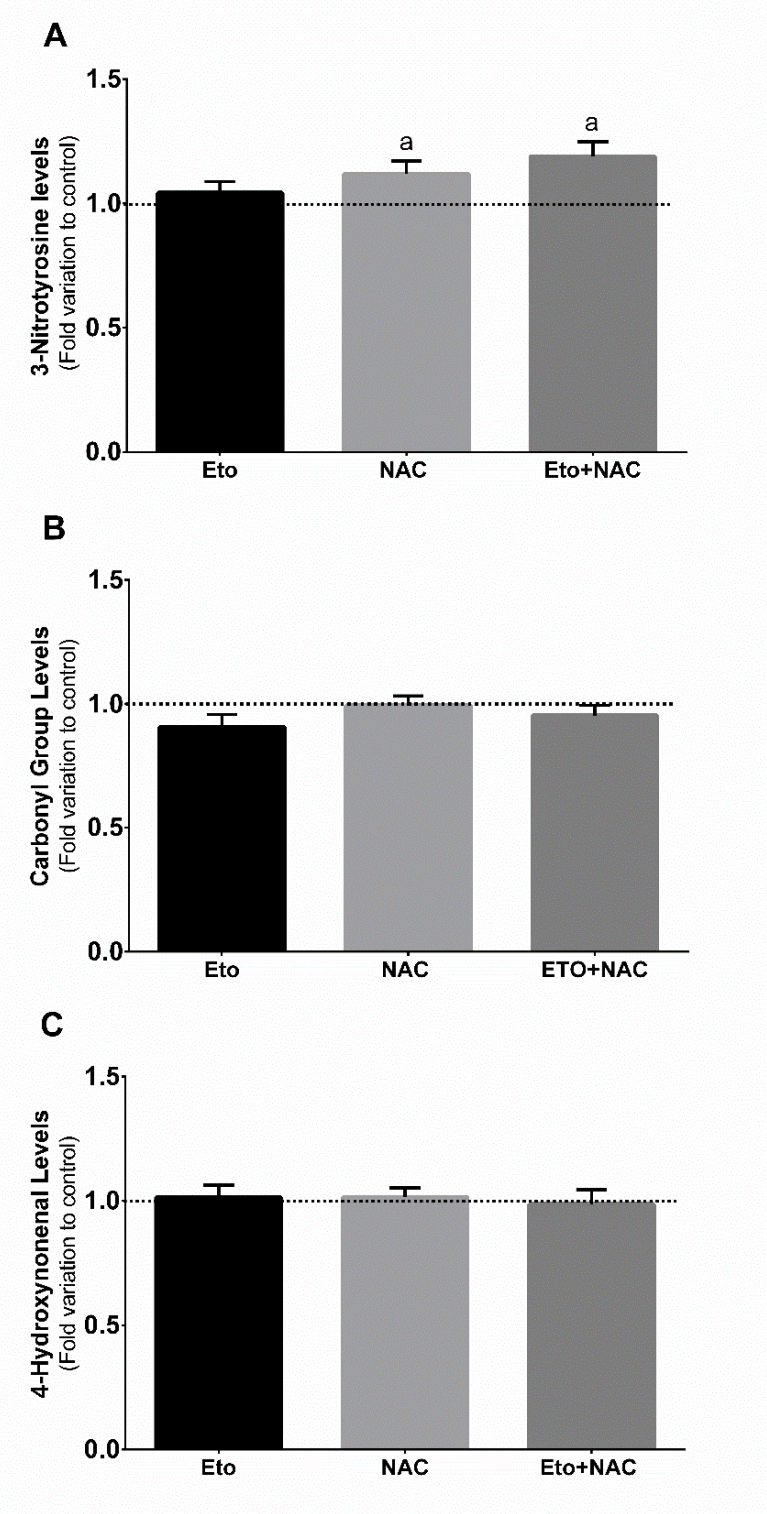

Figure III-3. Effect of exposure of human sperm to etoposide or/and NAC on oxidative stress markers. Protein nitration levels (panel A); protein carbonyl levels (panel B) and lipid peroxidation (panel C) were measured in human sperm after exposure to 25 µg/ml of etoposide (Eto), 50 µM of NAC (NAC) and both drugs in combination (Eto+NAC). Results are expressed in relation to the control group (Fold variation to control). Statistical significance was assessed by RM one-way ANOVA, followed by Fisher's LSD. Results are expressed as mean ± SEM (n=10 for each condition). Significantly different results ($p < 0.05$) are indicated as: a, relative to Eto.

3.7. Effects on sperm metabolism

Sperm metabolism and nutritional status play a crucial role in the overall reproductive capacity, and alterations in the metabolic profile of sperm are related to male factor infertility (*249*). The sperm preparation medium presents high amounts of glucose, which is essential for sperm capacitation and fertilization, being one of the preferential substances used by sperm for ATP production (*250*). As expected, our results showed that human sperm highly consumed glucose. In the control group glucose consumption was 32.0±5.2 pmol/10^6 sperm (Figure III-4A). No significant alterations were observed after incubation of human sperm with any of the agents tested. Nonetheless, sperm incubated with both drugs in combination revealed a non-significant increase of 1.5 fold in glucose consumption levels (Figure III-4A).

Pyruvate is other crucial substrate for sperm metabolism being responsible for the enhancement of glycolytic ATP production, sperm motility, capacitation and overall fertility status (*251*). Our results showed that pyruvate consumption was 10.6±2.0 pmol/10^6 sperm in the control group (Figure III-4B). Exposure of human sperm to etoposide or/and NAC did not alter pyruvate consumption in comparison to the control. Nevertheless, sperm incubated with Eto+NAC revealed a non-significant increase of 1.6 fold in pyruvate consumption levels (Figure III-4B). Although lactate was not present in sperm preparation medium it was accumulated in all groups. In the control group lactate production by human sperm was 11.2±1.0 pmol/10^6 sperm (Figure III-4C). Neither the exposure of sperm to etoposide, nor the exposure to NAC, or both drugs in combination altered lactate production (Figure III-4C).

Acetate is an important metabolite in fatty acids and cholesterol synthesis pathways (*252*). Our results showed that acetate production by human sperm was 0.001±0.001 pmol/10^6 sperm in the control group (Figure III-5A). After incubation with etoposide, NAC and a combination of both agents there were no significant differences in acetate production (Figure III-5A).

Choline is an important nutrient for sperm physiology, being a pivotal factor for sperm maturation and fertilization capacity (*253*). Furthermore, this nutrient is essential for sperm motility (*254*). Choline accumulation was 0.4±0.1 pmol/10^6 sperm in the control group (Figure III-5B). Similar levels were obtained in sperm incubated with etoposide (0.4±0.1 pmol/10^6 sperm). Interestingly, exposure of human sperm to NAC displayed a significant increase ($p=0.04$) in choline accumulation levels, 1.1±0.4 pmol/10^6 sperm, in relation to control group, etoposide and Eto+NAC groups. Notably, sperm exposed to both agents had the lowest levels of choline accumulation 0.1±0.1 pmol/10^6 sperm, a significant decrease in

relation to the sperm exposed to NAC (p=0.004) but not to control or etoposide groups (Figure III-5B).

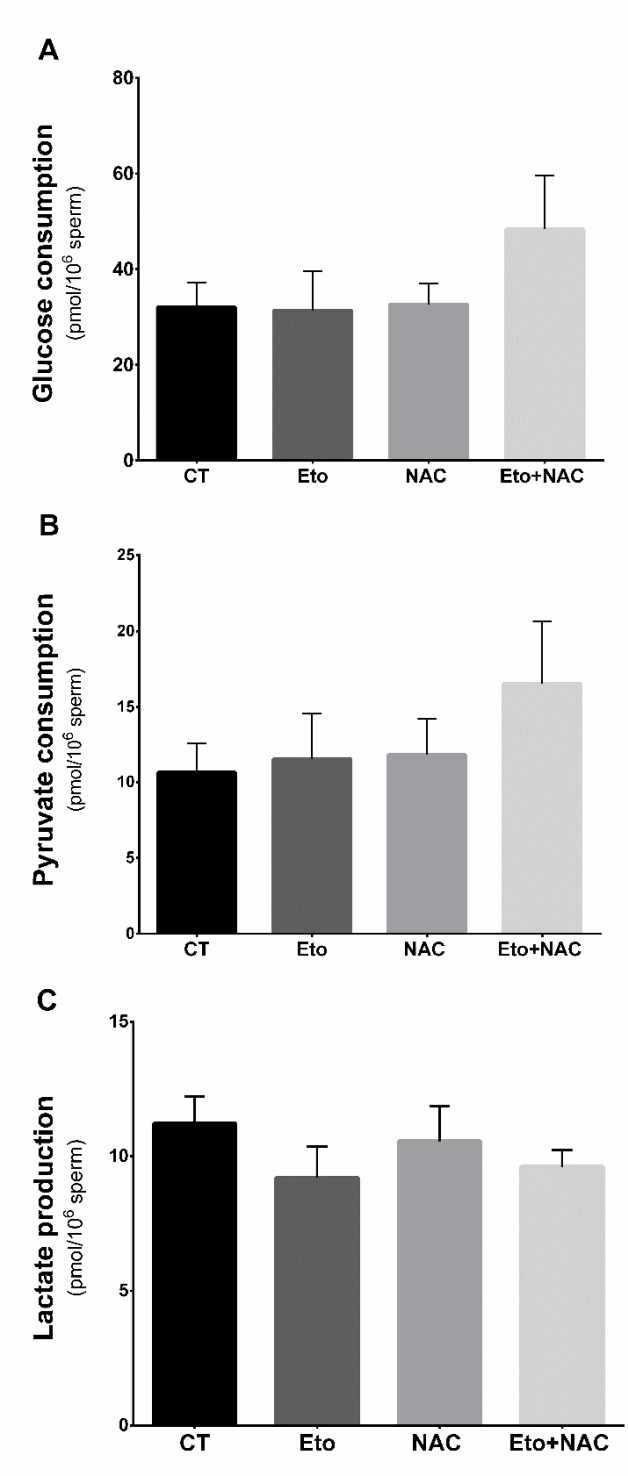

Figure III-4. Effect of exposure of human sperm to etoposide or/and NAC on glucose metabolism. Glucose consumption (panel A); pyruvate consumption (panel B) and lactate production (panel C) were measured in human sperm before (CT) and after exposure to 25 µg/ml of etoposide (Eto), 50 µM of NAC (NAC) and both drugs in combination (Eto+NAC). Statistical significance was assessed by RM one-way ANOVA, followed by Fisher's LSD. Results are expressed as mean ± SEM (n = 10 for each condition).

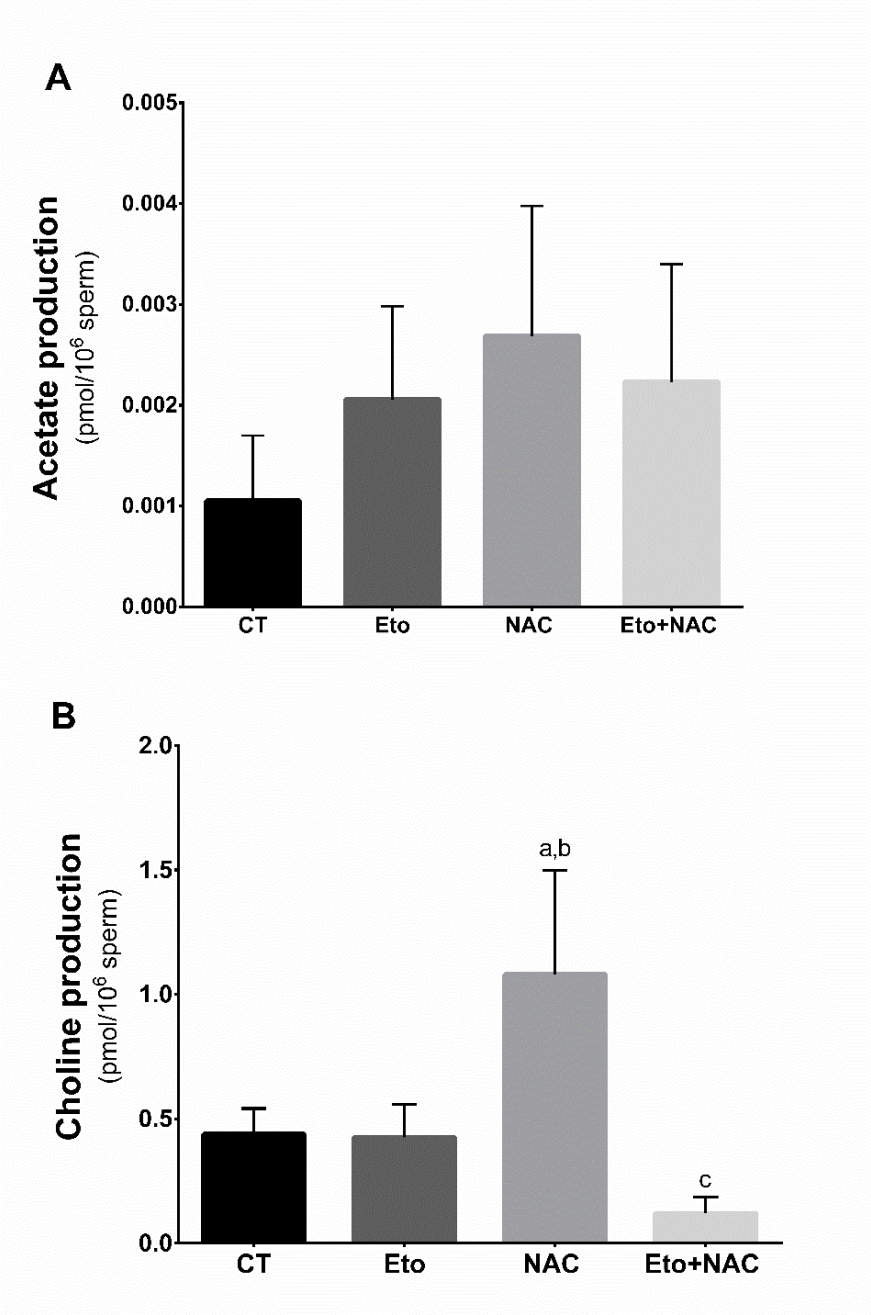

Figure III-5. Effect of exposure of human sperm etoposide or/and NAC on acetate and choline production. Acetate (panel A) and choline (panel B) production measured in human sperm before (CT) and after exposure to 25 µg/ml of etoposide (Eto), 50 µM of NAC (NAC) and both drugs in combination (Eto+NAC). Statistical significance was assessed by RM one-way ANOVA, followed by Fisher's LSD. Results are expressed as mean ± SEM (n = 10 for each condition). Significantly different results ($p < 0.05$) are indicated as: a, relative to control (CT); b, relative to Eto; c, relative to NAC.

.

4. Discussion

Etoposide is a chemotherapeutic drug regularly used as a first-line agent in the treatment of numerous neoplasia common in patients of reproductive age (*128*). It is a phase-specific, cytotoxic drug that inhibits DNA synthesis by forming a complex between topoisomerase II and DNA (*131*). Most chemotherapeutics are known as gonadotoxic though, most of their adverse effects on male reproduction are difficult to predict (*31*). In an effort to preserve healthy cells from chemotherapy-induced damages, various cytoprotective agents have been tested (*151*). NAC is a derivative of the amino acid L-cysteine (*189*) and its administration as supplement against several diseases is being tested. Some encouraging results have been reported due to its chemopreventive features and antioxidant activity (*192, 193*). Thus, we aimed to evaluate NAC's ability to preserve human sperm from the *in vitro* damaging effects induced by etoposide.

Our results show that *in vitro* exposure of human sperm to a pharmacological concentration (25 µg/ml) of etoposide does not significantly alter their motility, morphology and membrane integrity. It was previously reported that exposure to etoposide *in vitro* resulted in decreased sperm quality and viability (*128*). Accordingly, the evaluation of patients treated with etoposide-based therapies revealed a decrease in the overall spermatic quality after treatment (*255*). Moreover, patients treated with 100 mg/m^2 of etoposide for a 5 day period presented a low percentage of morphologically normal sperm (*118*). Although our results show that a 2 h exposure to etoposide is insufficient to significantly alter sperm motility, morphology and viability. The incubation of human sperm with NAC does not affect sperm motility and morphology, but reduces cell viability when compared to etoposide. Several studies in other cellular types have reported that NAC can have a pro-oxidant activity under certain conditions (*256, 257*). Pro-oxidant environments have been associated with a decrease in the overall spermatic quality, particularly cell viability (*247*), which would explain the NAC-induced deleterious effects on sperm viability found in our study.

In addition to sperm motility, viability and morphology, it is crucial to evaluate DNA integrity when assessing the overall sperm quality (*258*). Indeed, it is essential for male fertility to produce sperm with highly condensed and preserved DNA (*259*). A reduction in sperm quality after chemotherapeutic treatment is generally attributed to DNA damage (*260*). Our results show that a 2 h exposure of human sperm to etoposide is responsible for an increase in the percentage of sperm with immature chromatin condensation. Furthermore, etoposide is responsible for a high increase in DNA fragmentation levels and therefore, in the percentage of TUNEL positive sperm. Studies conducted in mice germ cells *in vitro* have showed that exposure to etoposide (5 mM for 6 h) results in vast DNA

damage and chromatin modifications (*261*). Moreover, it has been previously reported that treatment with etoposide therapeutic dosages (40-80 mg/kg) was responsible for an increase in the density of TUNEL positive cells in rats (*240*), and a high increase in sperm chromosomal aberrations (*260*) and DNA mutations (*262*) in mice. Our results are concomitant with these previous studies since they show that a 2 h exposure to etoposide induces DNA damage in sperm. Notably, sperm exposed to a combination of etoposide and NAC presented levels of immature chromatin condensation and DNA fragmentation similar to the control group, and lower than those observed in etoposide-exposed sperm, thus illustrating that NAC preserves DNA integrity of human sperm exposed to etoposide. Others have shown that NAC inhibits spontaneous and induced mutations, preventing DNA damage (*263, 264*). Studies have also reported that NAC has solid DNA protective effects, either by acting in the extracellular environment or by replenishing cellular antioxidant mechanisms (*265*). A study conducted by Lopes and colleagues has reported that the addition of NAC (0.1 mM) to sperm reduces DNA fragmentation induced by oxidative stress (*266*). The same was observed in other human cellular types, where 500 μM of NAC protected cells from oxidative stress-induced toxicity (*267*). Thus, our results show that 2 h exposure of human sperm to etoposide causes chromatin and DNA damage. The exposure to NAC alone does not significantly affect sperm DNA integrity. Nonetheless, the addition of NAC to etoposide shields human sperm DNA, particularly chromatin condensation and DNA fragmentation levels.

It has been hypothesised that etoposide induces DNA damage by ROS formation and the consequent induction of oxidative stress (*132*). Our results show that 2 h exposure of human sperm to etoposide does not alter the oxidative profile of these cells in relation to protein nitration and carbonylation and lipid peroxidation. An *in vitro* study with rat thymocytes exposed to 10 μM of etoposide for a 4 h period reported apoptosis induction probably due to hydroxyl radical formation (*268*). Others have also reported hydroxyl radical production by etoposide, which caused DNA nicking (*269*). Although these results are not in agreement with our findings, it should be noted that our experiment was performed in a 2 h exposure. Therefore, our results provide evidence that 2 h exposure of human sperm to etoposide does not induce major cellular oxidative damages. Our results also revealed that exposure of sperm to NAC alone does not alter the oxidative profile of sperm in relation to protein carbonylation and lipid peroxidation. Interestingly, human sperm incubated with NAC alone or in combination with etoposide displayed an increase in protein nitration. These findings are in agreement with the reduction in sperm viability observed after exposure to NAC and etoposide plus NAC. Several studies reported the antioxidant properties of NAC as the main mechanism for sperm protection and DNA fragmentation

reduction (*242, 266*). It was also reported that incubation of human semen with 1 mg/ml of NAC at room temperature significantly decreased ROS levels after 20 min (*270*). Nonetheless, this reduction was dose-dependent and greater in groups with initial higher ROS production. There is some controversy on the antioxidant and pro-oxidant properties of NAC, which may depend on the dose and exposure time. For instance, an *in vivo* study with mice has shown that NAC exerts a strong antioxidant defense in cells with increased nitrative stress (*271*). However, studies in other cellular types reported that NAC can have a pro-oxidant activity, generating hydrogen peroxide and increasing the secretion of nitric oxide and cellular nitrotyrosine (*256, 257*). Thus, our results suggest that, in our experimental conditions, NAC has pro-oxidant activity, increasing nitrotyrosine levels and reducing sperm viability in relation to sperm exposed to etoposide alone. It has been previously reported that etoposide acts by inhibiting topoisomerase II, an enzyme responsible for relaxing supercoiled DNA, forming a stable complex between topoisomerase II and DNA, which directly results in DNA strand break, cell cycle blockage and finally cellular death (*131*). Hence, our results provide evidence that etoposide directly affects sperm chromatin condensation and DNA fragmentation without oxidative stress-mediated mechanisms.

Sperm cells metabolism is essential to obtain the required energy for sperm regular functions, such as motility, being glycolysis the preferential pathway (*249*). Our results show that human sperm glucose consumption was maintained after exposure to etoposide. Moreover, sperm exposed to NAC alone or in combination with etoposide showed no significant alteration in glucose and pyruvate uptake, and lactate and acetate production. These results illustrate that the glycolytic profile of human sperm is not altered by 2 h exposure to etoposide, NAC or the combination of both drugs. Nevertheless, recent studies have reported that high glucose levels in culture medium protect cells from the deleterious effects of etoposide (*272*). These findings are in agreement with our results that show no decrease in sperm viability and motility after 2 h exposure to etoposide. To the best of our knowledge this is the first report on the effects of etoposide or/and NAC on glucose metabolism. Overall, our results provide evidence that neither etoposide nor NAC affect human sperm glycolytic profile. Interestingly, choline metabolism is associated with regulation of sperm membrane structure and fluidity, being crucial for sperm cells function (*253*). In fact, male choline dehydrogenase knockout mice (*Chdh*$^{-/-}$) are infertile (*254*). Our results show that exposure of human sperm to NAC alone is responsible for an increase in extracellular choline accumulation in relation to all groups. These results suggest that deregulated choline metabolism may be associated with the observed decrease in sperm

viability after exposure to NAC. Further studies are needed to unveil the relevance of choline metabolism and NAC to human sperm.

In conclusion, although 2 h exposure to etoposide does not affect sperm viability, it induces severe chromatin alterations and DNA damage to human sperm. These alterations may compromise male fertility and are potentially transmittable to the next generation (*273*). The addition of NAC to etoposide sperm exposure protected cellular chromatin integrity and reduced DNA fragmentation. Thus, it acts as a cytoprotector agent, shielding human sperm DNA from etoposide-induced damages. A 2 h exposure of human sperm to etoposide does not induce cellular oxidative damages nor glycolytic profile alterations, providing evidence that etoposide directly affects sperm DNA. NAC's ability to preserve human sperm from DNA damages induced after a 2 h exposure to etoposide may be of clinical relevance, as the majority of patients undergoing chemotherapy fail to collect semen samples prior to the initiation of treatment (*20*). NAC presents indeed great advantages as it is an inexpensive drug, already approved for safe human use, with minimal adverse effects. Thus, the integrity of the collected sperm after treatment initiation could be more easily preserved with the addition of NAC, guaranteeing that the majority of viable cells would have their DNA integrity preserved. Thus assuring that the future use of these patients' cryopreserved sperm in assisted reproductive techniques would not be compromised. Further studies are needed to effectively assess NAC's beneficial effects on human sperm during chemotherapy.

IV. Final Remarks

Final Remarks

Cancer treatment has been improving and survival rates are at their highest percentage ever. Chemotherapy secondary effects on male fertility are varied and it is difficult to predict the individual potential risk for persistent infertility. Thus, several fertility preservation options are being studied and must be debated with patients.

Our study on the damaging effects of etoposide on human spermatozoa and NAC's ability to preserve these cells from such damages provide evidence that the addition of NAC to sperm exposed to etoposide protects cellular chromatin integrity and reduces DNA fragmentation. Overall, our results show that the mechanism behind etoposide cytotoxic action relies on its direct interaction with DNA, not causing oxidative damages nor altering the glycolytic profile of human sperm. Furthermore, short-term exposure to etoposide does not affect sperm vitality, motility or morphology. Nonetheless, it induces severe chromatin alterations and DNA fragmentation, severely compromising male fertility.

According to our results, NAC addition to sperm exposed to etoposide protects sperm chromatin integrity and reduces DNA fragmentation. These findings suggest that NAC acts as a cytoprotector agent, shielding human sperm DNA from etoposide-induced damages. However, the mechanism behind NAC's ability to preserve human sperm from DNA damages induced after a short-term exposure to etoposide does not seem to rely on its antioxidant properties.

As the majority of patients initiating chemotherapy fails to collect semen samples prior to the beginning of treatment, NAC's ability to preserve sperm DNA is of clinical relevance. Indeed, NAC presents numerous advantages as it is an inexpensive drug, has already been approved for human use and has minimal side effects. Hence, DNA integrity of sperm collected after treatment initiation could be preserved with the addition of NAC, assuring the majority of viable spermatozoa would be suitable for future use in Medically Assisted Reproduction.

V. Future Perspectives

Future Perspectives

Although our results on NAC's aptitude as a protector agent of human spermatozoa against etoposide-induced damages are promising, supplementary research must be conducted to attest the mechanism involved in NAC ability to protect DNA. Moreover, it would be useful to measure cellular glutathione levels after etoposide and NAC administration to the cellular medium, and with both agents in combination, to validate our results that oxidative stress does not play a role in etoposide cytotoxicity mechanism. Likewise, further studies are needed to assess NAC's effects on human spermatozoa during long-term chemotherapeutic treatment.

Although our results on NAC's use against etoposide-induced damages to mature spermatozoa are promising, there are no certainties on its competence against other types of chemotherapeutic drugs. Therefore, it would be interesting to assess its cytoprotector abilities against other agents. Furthermore, as antioxidants have stronger effects on cells already under stress, it would be of value to administer NAC to cells that have been previously exposed to chemotherapeutics. Although this would not have direct benefits on spermatozoa already produced in the first chemotherapeutic regimen, it could be beneficial to preserve spermatogonia and assure the future maintenance of spermatogenesis.

Additionally, as antioxidants alone can have pro-oxidant effects, and in combination can have strong antioxidant activities, it would be interesting to expose cells to NAC in combination with other powerful antioxidants and assess their ability to fully preserve spermatozoa, already produced at the beginning of chemotherapy, and other cells of the spermatogenic line, assuring the maintenance of spermatogenesis.

VI. References

References

1. E. Steliarova-Foucher *et al.*, Geographical patterns and time trends of cancer incidence and survival among children and adolescents in Europe since the 1970s (the ACCIS project): an epidemiological study. *The Lancet* **364**, 2097-2105 (2004).
2. W. H. B. Wallace, R. A. Anderson, D. S. Irvine, Fertility preservation for young patients with cancer: who is at risk and what can be offered? *The Lancet Oncology* **6**, 209-218 (2005).
3. G. R. Dohle, Male infertility in cancer patients: Review of the literature. *Int. J. Urol.* **17**, 327-331 (2010).
4. J. P. Ginsberg, Educational paper: the effect of cancer therapy on fertility, the assessment of fertility and fertility preservation options for pediatric patients. *Eur. J. Pediatr.* **170**, 703-708 (2011).
5. K. Jahnukainen, J. Ehmcke, M. Hou, S. Schlatt, Testicular function and fertility preservation in male cancer patients. *Best Pract. Res. Clin. Endocrinol. Metab.* **25**, 287-302 (2011).
6. S. J. Howell, S. M. Shalet, Pharmacological Protection of the Gonads. *Med. Pediatr. Oncol.* **33**, 41–45 (1999).
7. C. Gonzalez, M. Boada, M. Devesa, A. Veiga, Concise review: fertility preservation: an update. *Stem Cells Transl Med* **1**, 668-672 (2012).
8. L. I. Lipshultz, S. S. Howards, C. S. Niederberger, *Infertility in the Male*. (Cambridge University Press, ed. 4th, 2009), pp. 654.
9. D. G. Rooij, L. D. Russell, All You Wanted to Know About Spermatogonia but Were Afraid to Ask. *J. Androl.* , (2000).
10. R. E. Brannigan *et al.*, *An Introduction to Male Reproductive Medicine*. (Cambridge University Press, New York, United States of America 2011), pp. 152.
11. R. A. Hess, L. R. Franca, in *Molecular Mechanisms in Spermatogenesis,* C. Y. Cheng, Ed. (Landes Bioscience and Springer Science+Business Media, 2008), chap. 1.
12. D. H. t. Williams, Fertility preservation in the male with cancer. *Curr. Urol. Rep.* **14**, 315-326 (2013).
13. M. L. Meistrich, Effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertil. Steril.* **100**, 1180-1186 (2013).
14. S. Howell, S. Shalet, Gonadal damage from chemotherapy and radiotherapy. *Endocrinol. Metab. Clin. North Am.* **27**, 927-943 (1998).
15. H. Magelssen, M. Brydoy, S. D. Fossa, The effects of cancer and cancer treatments on male reproductive function. *Nat. Clin. Pract. Urol.* **3**, 312-322 (2006).
16. T. Shinohara *et al.*, Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. *Hum. Reprod.* **17**, 3039-3045 (2002).
17. S. Schlatt, J. Ehmcke, K. Jahnukainen, Testicular stem cells for fertility preservation: preclinical studies on male germ cell transplantation and testicular grafting. *Pediatr. Blood Cancer* **53**, 274-280 (2009).
18. R. Sa, N. Cremades, I. Malheiro, M. Sousa, Cryopreservation of human testicular diploid germ cell suspensions. *Andrologia* **44**, 366-372 (2012).
19. J. M. Hotaling *et al.*, Raw and test-thaw semen parameters after cryopreservation among men with newly diagnosed cancer. *Fertil. Steril.* **99**, 464-469 (2013).
20. M. Trottmann *et al.*, Semen quality in men with malignant diseases before and after therapy and the role of cryopreservation. *Eur. Urol.* **52**, 355-367 (2007).
21. Devita, Hellman, Rosenberg, *Cancer - Principles And Practice Of Oncology* (Lippincott Williams & Wilkins Publishers, ed. 6th, 2001).

22. M. Ethics Committee of American Society for Reproductive, Fertility preservation and reproduction in patients facing gonadotoxic therapies: a committee opinion. *Fertil. Steril.* **100**, 1224-1231 (2013).
23. R. Tal *et al.*, Follow-up of sperm concentration and motility in patients with lymphoma. *Hum. Reprod.* **15** 1985-1988 (2000).
24. R. D. van Beek *et al.*, Inhibin B is superior to FSH as a serum marker for spermatogenesis in men treated for Hodgkin's lymphoma with chemotherapy during childhood. *Hum. Reprod.* **22**, 3215-3222 (2007).
25. E. M. Rinchick *et al.*, Genetic and Molecular analysis of chlorambucil-induced germ-line mutations in the mouse. *Genetics*, 1416-1420 (1990).
26. W. M. Generoso *et al.*, Dominant lethal and heritable translocation tests with chlorambucil and melphalan in male mice. *Mutat. Res.* **345**, 167-180 (1995).
27. A. L. Drumond *et al.*, Effects of multiple doses of cyclophosphamide on mouse testes: accessing the germ cells lost, and the functional damage of stem cells. *Reprod. Toxicol.* **32**, 395-406 (2011).
28. R. M. Pryzant, M. L. Meistrich, G. Wilson, B. Brown, P. McLaughlin, Long-term reduction in sperm count after chemotherapy with and without radiation therapy for non-Hodgkin's lymphomas. *J. Clin. Oncol.* **11**, 239-247 (1993).
29. A. Aguilar-Mahecha, B. F. Hales, B. Robaire, Effects of acute and chronic cyclophosphamide treatment on meiotic progression and the induction of DNA double-strand breaks in rat spermatocytes. *Biol. Reprod.* **72**, 1297-1304 (2005).
30. P. Ypsilantis, N. Papaioannoub, D. Psalla, M. Politou, C. Simopoulosa, Effects of single dose administration of ifosfamide on testes and semen characteristics in the rabbit. *Reprod. Toxicol.*, 237–245 (2002).
31. T. Ishikawa, S. Kamidono, M. Fujisawa, Fertility after high-dose chemotherapy for testicular cancer. *Urology*, 137–140 (2004).
32. L. R. Bucci, M. L. Meistrich, Effects of busulfan on murine spermatogenesis: cytotoxicity, sterility, sperm abnormalities, and dominant lethal mutations. *Mutat. Res.*, 259-268 (1987).
33. M. Mirhoseini, G. Saki, M. Hemadi, A. Khodadadi, J. Mohammadi Asl, Melatonin and testicular damage in busulfan treated mice. *Iran Red Crescent Med J* **16**, e14463 (2014).
34. M. L. Meistrich, M. Finch, M. F. Cunha, U. Hacker, W. W. Au, Damaging Effects of Fourteen Chemotherapeutic Drugs on Mouse Testis Cells. *Cancer Res.* **42**, 122-131 (1982).
35. A. D. Tates, A. T. Natarajan, N. De Vogel, M. Meijers, A correlative study of the genetic damage induced by chemical mutagens in bone marrow and spermatogonia of mice. III. 1,3-Bis (2-chloroethyl)-3-nitrosourea (BCNU). *Mutat. Res.* **44**, 87-95 (1977).
36. U. H. Ehling, I. D. Adler, J. Favor, A. Neuhauser-Klaus, Induction of specific-locus and dominant lethal mutations in male mice by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). *Mutat. Res.* **379**, 219-231 (1997).
37. D. J. Thompson, J. A. Molello, R. J. Strebing, I. L. Dyke, V. B. Robinson, Reproduction and teratology studies with oncolytic agents in the rat and rabbit. I. I,3-bis(2-chloroethyl)-i-nitrosourea (BCNU). *Toxicol. Appl. Pharmacol.* **30**, 422-439 (1974).
38. P. Kopf-Maier, Effects of carboplatin on the testis. A histological study. *Cancer Chemother. Pharmacol.* **29**, 227-235 (1992).
39. H. Fuse, Y. Fujiuchi, A. Junicho, M. Iwasaki, T. Katayama, Effect of carboplatin on rat spermatogenesis. *Urol. Int.* **56**, 219-223 (1996).
40. W. J. Reiter *et al.*, Sperm analysis and serum follicle-stimulating hormone levels before and after adjuvant single-agent carboplatin therapy for clinical stage I seminoma. *Urology* **52**, 117-119 (1998).
41. W. J. Reiter, S. Tomek, C. C. Zielinski, M. Marberger, Effect of carboplatin on the functional integrity of the human sperm membrane in vitro. *J. Androl.* **23**, 338-340 (2002).

42. S. Puyo, D. Montaudon, P. Pourquier, From old alkylating agents to new minor groove binders. *Crit. Rev. Oncol. Hematol.* **89**, 43-61 (2014).
43. F. Drablos *et al.*, Alkylation damage in DNA and RNA--repair mechanisms and medical significance. *DNA Repair (Amst)* **3**, 1389-1407 (2004).
44. B. Kaina, M. Christmann, S. Naumann, W. P. Roos, MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair (Amst)* **6**, 1079-1099 (2007).
45. F. M. Schabel, Jr. *et al.*, Experimental evaluation of potential anticancer agents VIII. Effects of certain nitrosoureas on intracerebral L1210 leukemia. *Cancer Res.* **23**, 725-733 (1963).
46. M. L. Meistrich, Male gonadal toxicity. *Pediatr. Blood Cancer* **53**, 261-266 (2009).
47. W. J. van der Vijgh, Clinical pharmacokinetics of carboplatin. *Clin. Pharmacokinet.* **21**, 242-261 (1991).
48. S. B. Duffull, B. A. Robinson, Clinical pharmacokinetics and dose optimisation of carboplatin. *Clin. Pharmacokinet.* **33**, 161-183 (1997).
49. A. Rabbani, R. M. Finn, J. Ausio, The anthracycline antibiotics: antitumor drugs that alter chromatin structure. *Bioessays* **27**, 50-56 (2005).
50. B. R. Abdella, J. Fisher, A chemical perspective on the anthracycline antitumor antibiotics. *Environ. Health Perspect.* **64**, 4-18 (1985).
51. G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, L. Gianni, Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **56**, 185-229 (2004).
52. H. Takahashi *et al.*, Evaluation of testicular toxicology of doxorubicin based on microarray analysis of testicular specific gene expression. *J. Toxicol. Sci.* **36**, 559-567 (2011).
53. V. Vendramini, B. Robaire, S. M. Miraglia, Amifostine-doxorubicin association causes long-term prepubertal spermatogonia DNA damage and early developmental arrest. *Hum. Reprod.* **27**, 2457-2466 (2012).
54. O. Brilhante, F. K. Okada, E. Sasso-Cerri, T. Stumpp, S. M. Miraglia, Late morfofunctional alterations of the Sertoli cell caused by doxorubicin administered to prepubertal rats. *Reprod. Biol. Endocrinol.* **10**, 79 (2012).
55. J. Gaffan *et al.*, Infertility rates following POMB/ACE chemotherapy for male and female germ cell tumours - a retrospective long-term follow-up study. *Br. J. Cancer* **89**, 1849-1854 (2003).
56. D. Chan, G. Delbes, M. Landry, B. Robaire, J. M. Trasler, Epigenetic alterations in sperm DNA associated with testicular cancer treatment. *Toxicol. Sci.* **125**, 532-543 (2012).
57. L. Marcon, X. Zhang, B. F. Hales, B. Robaire, M. C. Nagano, Effects of chemotherapeutic agents for testicular cancer on rat spermatogonial stem/progenitor cells. *J. Androl.* **32**, 432-443 (2011).
58. J. Maselli, B. F. Hales, P. Chan, B. Robaire, Exposure to bleomycin, etoposide, and cis-platinum alters rat sperm chromatin integrity and sperm head protein profile. *Biol. Reprod.* **86**, 166, 161-110 (2012).
59. T. Amirshahi, G. Najafi, V. Nejati, Protective effect of royal jelly on fertility and biochemical parameters in bleomycin-induced male rats. *Iran J Reprod Med* **12**, 209-216 (2014).
60. L. Bujan *et al.*, Impact of lymphoma treatments on spermatogenesis and sperm deoxyribonucleic acid: a multicenter prospective study from the CECOS network. *Fertil. Steril.* **102**, 667-674 e663 (2014).
61. M. Liu, B. F. Hales, B. Robaire, Effects of four chemotherapeutic agents, bleomycin, etoposide, cisplatin, and cyclophosphamide, on DNA damage and telomeres in a mouse spermatogonial cell line. *Biol. Reprod.* **90**, 72 (2014).
62. A. B. Edgar, W. H. B. Wallace, Reproductive possibilities for the child with cancer. *Obstetrics, Gynaecology and Reproductive Medicine* **19**, 48-52 (2008).

63. P. A. Speth, Q. G. van Hoesel, C. Haanen, Clinical pharmacokinetics of doxorubicin. *Clin. Pharmacokinet.* **15**, 15-31 (1988).
64. T. Yokochi, K. D. Robertson, Doxorubicin inhibits DNMT1, resulting in conditional apoptosis. *Mol. Pharmacol.* **66**, 1415-1420 (2004).
65. E. Reich, R. M. Franklin, A. J. Shatkin, Tatumel, Action of actinomycin D on animal cells and viruses. *Proc. Natl. Acad. Sci. U. S. A.* **48**, 1238-1245 (1962).
66. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, Effect of actinomycin D on cellular nucleic acid synthesis and virus production. *Science* **134**, 556-557 (1961).
67. C. R. Hill *et al.*, Characterisation of the clinical pharmacokinetics of actinomycin D and the influence of ABCB1 pharmacogenetic variation on actinomycin D disposition in children with cancer. *Clin. Pharmacokinet.* **53**, 741-751 (2014).
68. W. J. Barcellona, R. B. Brackeen, B. R. Brinkley, Differential binding of tritiated actinomycin to the nuclei of mammalian spermatogenic cells in vivo. *J. Reprod. Fertil.* **39**, 41-48 (1974).
69. D. S. Alberts *et al.*, Bleomycin pharmacokinetics in man. I. Intravenous administration. *Cancer Chemother. Pharmacol.* **1**, 177-181 (1978).
70. W. G. Kramer *et al.*, The pharmacokinetics of bleomycin in man. *J. Clin. Pharmacol.* **18**, 346-352 (1978).
71. L. M. Mir, O. Tounekti, S. Orlowski, Bleomycin: revival of an old drug. *Gen. Pharmacol.* **27**, 745-748 (1996).
72. G. J. Peters *et al.*, Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol. Ther.* **87**, 227-253 (2000).
73. P. D. Cole, J. A. Zebala, B. A. Kamen, Antimetabolites: A new perspective. *Drug Discovery Today: Therapeutic Strategies*. 2005 (10.1016/j.ddstr.2005.11.004).
74. P. Frost, B. A. Kamen, The bullseye of cancer therapy: a moving target. *Curr. Opin. Pharmacol.* **3**, 335-337 (2003).
75. S. B. Kaye, New antimetabolites in cancer chemotherapy and their clinical impact. *Br. J. Cancer* **78 Suppl 3**, 1-7 (1998).
76. A. K. Palo, D. Sahoo, R. C. Choudhury, Cytosine arabinoside-induced cytogenotoxicity in bone marrow and spermatogonial cells of mice and its potential transmission through the male germline. *Mutat. Res.* **673**, 29-36 (2009).
77. K. D. Beaula Helen, S. Subramanyam, Genotoxic evaluation of Ara-C by multiple parameters. *Mutat. Res.* **263**, 185-196 (1991).
78. R. C. Namoju *et al.*, Prepubertal exposure of cytarabine induced testicular atrophy, impaired spermatogenesis and germ cell DNA damage in SD rats. *Toxicol. Mech. Methods*, 1-35 (2014).
79. R. C. Choudhury, S. Misra, M. B. Jagdale, A. K. Palo, Induction and transmission of cytogenetic toxic effects of 5-fluorouracil in male germline cells of Swiss mice. *J. Exp. Clin. Cancer Res.* **21**, 277-282 (2002).
80. U. J. D'Souza, Toxic effects of 5-Fluorouracil on sperm count in wistar rats. *Malays J Med Sci* **10**, 43-45 (2003).
81. U. J. D'Souza, K. Narayana, Induction of seminiferous tubular atrophy by single dose of 5-fluorouracil (5-FU) in Wistar rats. *Indian J. Physiol. Pharmacol.* **45**, 87-94 (2001).
82. M. Ligumsky, S. Badaan, H. Lewis, D. Meirow, Effects of 6-mercaptopurine treatment on sperm production and reproductive performance: a study in male mice. *Scand. J. Gastroenterol.* **40**, 444-449 (2005).
83. Z. S. Heetun, C. Byrnes, P. Neary, C. O'Morain, Review article: Reproduction in the patient with inflammatory bowel disease. *Aliment. Pharmacol. Ther.* **26**, 513-533 (2007).
84. R. O. Rajapakse, B. I. Korelitz, J. Zlatanic, P. J. Baiocco, G. W. Gleim, Outcome of pregnancies when fathers are treated with 6-mercaptopurine for inflammatory bowel disease. *Am. J. Gastroenterol.* **95**, 684-688 (2000).

85. S. V. Kane, What's good for the goose should be good for the gander--6-MP use in fathers with inflammatory bowel disease. *Am. J. Gastroenterol.* **95**, 581-582 (2000).
86. R. C. Choudhury, S. K. Ghosh, A. K. Palo, Potential transmission of the cytogenetic toxic effects of methotrexate in the male germline cells of Swiss mice. *Environ Toxicol Pharmacol* **10**, 81-88 (2001).
87. S. Padmanabhan, D. N. Tripathi, A. Vikram, P. Ramarao, G. B. Jena, Methotrexate-induced cytotoxicity and genotoxicity in germ cells of mice: intervention of folic and folinic acid. *Mutat. Res.* **673**, 43-52 (2009).
88. A. K. Palo, R. C. Choudhury, Modulation of methotrexate-induced cytogenotoxicity in mouse spermatogonia and its transmission in the male germline by caffeine. *Environ Toxicol Pharmacol* **21**, 254-259 (2006).
89. S. Padmanabhan, D. N. Tripathi, A. Vikram, P. Ramarao, G. B. Jena, Cytotoxic and genotoxic effects of methotrexate in germ cells of male Swiss mice. *Mutat. Res.* **655**, 59-67 (2008).
90. H. G. Oufi, N. N. Al-Shawi, The effects of different doses of silibinin in combination with methotrexate on testicular tissue of mice. *Eur. J. Pharmacol.* **730**, 36-40 (2014).
91. F. E. Johnson, S. A. Farr, M. Mawad, Y. C. Woo, Testicular cytotoxicity of intravenous methotrexate in rats. *J. Surg. Oncol.* **55**, 175-178 (1994).
92. I. Sukhotnik *et al.*, Methotrexate induces germ cell apoptosis and impairs spermatogenesis in a rat. *Pediatr. Surg. Int.* **29**, 179-184 (2013).
93. M. Koehler, R. Waldherr, R. Ludwig, U. Heinrich, W. E. Brandeis, Effects of methotrexate on rabbit testes. Part 1: Morphological changes. *Pediatr. Hematol. Oncol.* **3**, 325-334 (1986).
94. L. F. Morris, M. J. Harrod, M. A. Menter, A. K. Silverman, Methotrexate and reproduction in men: case report and recommendations. *J. Am. Acad. Dermatol.* **29**, 913-916 (1993).
95. F. Lamboglia, R. D'Inca, L. Oliva, P. Bertomoro, G. C. Sturniolo, Patient with severe Crohn's disease became a father while on methotrexate and infliximab therapy. *Inflamm. Bowel Dis.* **15**, 648-649 (2009).
96. S. Grant, Ara-C: cellular and molecular pharmacology. *Adv. Cancer Res.* **72**, 197-233 (1998).
97. S. Hatse, E. De Clercq, J. Balzarini, Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation. *Biochem. Pharmacol.* **58**, 539-555 (1999).
98. J. S. Kovach, R. W. Beart, Jr., Cellular pharmacology of fluorinated pyrimidines in vivo in man. *Invest. New Drugs* **7**, 13-25 (1989).
99. R. B. Diasio, B. E. Harris, Clinical pharmacology of 5-fluorouracil. *Clin. Pharmacokinet.* **16**, 215-237 (1989).
100. L. Lennard, D. Keen, J. S. Lilleyman, Oral 6-mercaptopurine in childhood leukemia: parent drug pharmacokinetics and active metabolite concentrations. *Clin. Pharmacol. Ther.* **40**, 287-292 (1986).
101. L. Lennard, The clinical pharmacology of 6-mercaptopurine. *Eur. J. Clin. Pharmacol.* **43**, 329-339 (1992).
102. S. Kivity *et al.*, Clinical characteristics and risk factors for low dose methotrexate toxicity: A cohort of 28 patients. *Autoimmun. Rev.*, (2014).
103. L. Genestier, R. Paillot, L. Quemeneur, K. Izeradjene, J. P. Revillard, Mechanisms of action of methotrexate. *Immunopharmacology* **47**, 247-257 (2000).
104. A. E. French, G. Koren, T. Motherisk, Effect of methotrexate on male fertility. *Can. Fam. Physician* **49**, 577-578 (2003).
105. E. S. Chan, B. N. Cronstein, Mechanisms of action of methotrexate. *Bull Hosp Jt Dis (2013)* **71 Suppl 1**, S5-8 (2013).
106. G. M. Cragg, D. J. Newman, Plants as a source of anti-cancer agents. *J. Ethnopharmacol.* **100**, 72-79 (2005).

107. D. J. Newman, G. M. Cragg, K. M. Snader, Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* **66**, 1022-1037 (2003).
108. S. C. D'Cruz, S. Vaithinathan, R. Jubendradass, P. P. Mathur, Effects of plants and plant products on the testis. *Asian J. Androl.* **12**, 468-479 (2010).
109. G. C. Jagetia, H. Krishnamurthy, P. Jyothi, Evaluation of cytotoxic effects of different doses of vinblastine on mouse spermatogenesis by flow cytometry. *Toxicology* **112**, 227-236 (1996).
110. P. Geriyol, H. B. Basavanneppa, B. L. Dhananjaya, Protecting effect of caffeine against vinblastine (an anticancer drug) induced genotoxicity in mice. *Drug Chem. Toxicol.*, 1-8 (2014).
111. W. A. Robbins *et al.*, Chemotherapy induces transient sex chromosomal and autosomal aneuploidy in human sperm. *Nat. Genet.* **16**, 74-78 (1997).
112. R. A. Cooke, A. Nikles, H. P. Roeser, A comparison of the antifertility effects of alkylating agents and vinca alkaloids in male rats. *Br. J. Pharmacol.* **63**, 677-681 (1978).
113. W. L. Hobbie, J. P. Ginsberg, S. K. Ogle, C. A. Carlson, A. T. Meadows, Fertility in males treated for Hodgkins disease with COPP/ABV hybrid. *Pediatr. Blood Cancer* **44**, 193-196 (2005).
114. A. Grigg, The impact of conventional and high-dose therapy for lymphoma on fertility. *Clin. Lymphoma* **5**, 84-88 (2004).
115. J. Rautonen, A. I. Koskimies, M. A. Siimes, Vincristine is associated with the risk of azoospermia in adult male survivors of childhood malignancies. *Eur. J. Cancer* **28A**, 1837-1841 (1992).
116. T. Stumpp, E. Sasso-Cerri, E. Freymuller, S. M. Miraglia, Apoptosis and testicular alterations in albino rats treated with etoposide during the prepubertal phase. *Anat. Rec. A. Discov. Mol. Cell. Evol. Biol.* **279**, 611-622 (2004).
117. J. A. Radford, S. Clark, D. Crowther, S. M. Shalet, Male fertility after VAPEC-B chemotherapy for Hodgkin's disease and non-Hodgkin's lymphoma. *Br. J. Cancer* **69**, 379-381 (1994).
118. W. T. Stephenson, S. M. Poirier, L. Rubin, L. H. Einhorn, Evaluation of reproductive capacity in germ cell tumor patients following treatment with cisplatin, etoposide, and bleomycin. *J. Clin. Oncol.* **13**, 2278-2280 (1995).
119. R. H. Martin *et al.*, Chromosomal abnormalities in sperm from testicular cancer patients before and after chemotherapy. *Hum. Genet.* **99**, 214-218 (1997).
120. P. De Mas *et al.*, Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin. *Hum. Reprod.* **16**, 1204-1208 (2001).
121. I. Har-Vardi *et al.*, DNA topoisomerases I and II in human mature sperm cells: characterization and unique properties. *Hum. Reprod.* **22**, 2183-2189 (2007).
122. M. S. Joshi, R. Y. Ambaye, Effect of alkaloids from Vinca rosea L. on spermatogenesis in male rats. *Indian J. Exp. Biol.* **6**, 256-257 (1968).
123. M. A. Jordan, D. Thrower, L. Wilson, Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res.* **51**, 2212-2222 (1991).
124. T. Sjoblom, M. Parvinen, J. Lahdetie, Stage-specific DNA synthesis of rat spermatogenesis as an indicator of genotoxic effects of vinblastine, mitomycin C and ionizing radiation on rat spermatogonia and spermatocytes. *Mutat. Res.* **331**, 181-190 (1995).
125. R. L. Nelson, The comparative clinical pharmacology and pharmacokinetics of vindesine, vincristine, and vinblastine in human patients with cancer. *Med. Pediatr. Oncol.* **10**, 115-127 (1982).
126. J. A. Silverman, L. Reynolds, S. R. Deitcher, Pharmacokinetics and pharmacodynamics of vincristine sulfate liposome injection (VSLI) in adults with acute lymphoblastic leukemia. *J. Clin. Pharmacol.* **53**, 1139-1145 (2013).

127. K. R. Hande, Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur. J. Cancer* **34**, 1514-1521 (1998).
128. W. M. Liu, S. P. Joel, The schedule-dependent effects of etoposide in leukaemic cell lines: a function of concentration and duration. *Cancer Chemother. Pharmacol.* **51**, 291-296 (2003).
129. P. Martinsson, S. Ekelund, P. Nygren, R. Larsson, The combination of the antitumoural pyridyl cyanoguanidine CHS 828 and etoposide in vitro--from cytotoxic synergy to complete inhibition of apoptosis. *Br. J. Pharmacol.* **137**, 568-573 (2002).
130. A. K. Larsen, A. E. Escargueil, A. Skladanowski, Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol. Ther.* **99**, 167-181 (2003).
131. S. M. Attia, Dominant lethal mutations of topoisomerase II inhibitors etoposide and merbarone in male mice: a mechanistic study. *Arch. Toxicol.* **86**, 725-731 (2012).
132. M. L. Slevin, The clinical pharmacology of etoposide. *Cancer* **67**, 319-329 (1991).
133. C. Wyns, M. Curaba, B. Vanabelle, A. Van Langendonckt, J. Donnez, Options for fertility preservation in prepubertal boys. *Hum. Reprod. Update* **16**, 312-328 (2010).
134. C. Di Bisceglie *et al.*, Effects of oncological treatments on semen quality in patients with testicular neoplasia or lymphoproliferative disorders. *Asian J. Androl.* **15**, 425-429 (2013).
135. J. P. Ginsberg, New advances in fertility preservation for pediatric cancer patients. *Curr. Opin. Pediatr.* **23**, 9-13 (2011).
136. Y. Chen, P. Jungsuwadee, M. Vore, D. A. Butterfield, D. K. St Clair, Collateral damage in cancer chemotherapy: oxidative stress in nontargeted tissues. *Mol. Interv.* **7**, 147-156 (2007).
137. J. Fang, T. Seki, H. Maeda, Therapeutic strategies by modulating oxygen stress in cancer and inflammation. *Adv Drug Deliv Rev* **61**, 290-302 (2009).
138. E. Geva, J. B. Lessing, L. Lerner-Geva, A. Amit, Free radicals, antioxidants and human spermatozoa: clinical implications. *Hum. Reprod.* **13**, 1422-1424 (1998).
139. A. Agarwal, K. P. Nallella, S. S. Allamaneni, T. M. Said, Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod. Biomed. Online* **8**, 616-627 (2004).
140. J. C. Kefer, A. Agarwal, E. Sabanegh, Role of antioxidants in the treatment of male infertility. *Int. J. Urol.* **16**, 449-457 (2009).
141. D. N. Tripathi, G. B. Jena, Astaxanthin inhibits cytotoxic and genotoxic effects of cyclophosphamide in mice germ cells. *Toxicology* **248**, 96-103 (2008).
142. A. Atessahin *et al.*, Protective role of lycopene on cisplatin-induced changes in sperm characteristics, testicular damage and oxidative stress in rats. *Reprod. Toxicol.* **21**, 42-47 (2006).
143. G. Turk, A. Atessahin, M. Sonmez, A. Yuce, A. O. Ceribasi, Lycopene protects against cyclosporine A-induced testicular toxicity in rats. *Theriogenology* **67**, 778-785 (2007).
144. G. Turk, A. O. Ceribasi, E. Sahna, A. Atessahin, Lycopene and ellagic acid prevent testicular apoptosis induced by cisplatin. *Phytomedicine* **18**, 356-361 (2011).
145. G. Turk, M. Sonmez, A. O. Ceribasi, A. Yuce, A. Atessahin, Attenuation of cyclosporine A-induced testicular and spermatozoal damages associated with oxidative stress by ellagic acid. *Int. Immunopharmacol.* **10**, 177-182 (2010).
146. A. O. Ceribasi, F. Sakin, G. Turk, M. Sonmez, A. Atessahin, Impact of ellagic acid on adriamycin-induced testicular histopathological lesions, apoptosis, lipid peroxidation and sperm damages. *Exp. Toxicol. Pathol.* **64**, 717-724 (2012).
147. G. Turk, A. Atessahin, M. Sonmez, A. O. Ceribasi, A. Yuce, Improvement of cisplatin-induced injuries to sperm quality, the oxidant-antioxidant system, and the histologic structure of the rat testis by ellagic acid. *Fertil. Steril.* **89**, 1474-1481 (2008).
148. E. Selvakumar, C. Prahalathan, P. T. Sudharsan, P. Varalakshmi, Chemoprotective effect of lipoic acid against cyclophosphamide-induced changes in the rat sperm. *Toxicology* **217**, 71-78 (2006).

149. E. Selvakumar, C. Prahalathan, Y. Mythili, P. Varalakshmi, Beneficial effects of DL-alpha-lipoic acid on cyclophosphamide-induced oxidative stress in mitochondrial fractions of rat testis. *Chem. Biol. Interact.* **152**, 59-66 (2005).
150. C. Prahalathan, E. Selvakumar, P. Varalakshmi, Protective effect of lipoic acid on adriamycin-induced testicular toxicity. *Clin. Chim. Acta* **360**, 160-166 (2005).
151. E. A. Ahmed, H. M. Omar, S. elghaffar, S. M. Ragb, A. Y. Nasser, The antioxidant activity of vitamin C, DPPD and L-cysteine against Cisplatin-induced testicular oxidative damage in rats. *Food Chem. Toxicol.* **49**, 1115-1121 (2011).
152. A. Chabra, M. Shokrzadeh, F. Naghshvar, F. Salehi, A. Ahmadi, Melatonin ameliorates oxidative stress and reproductive toxicity induced by cyclophosphamide in male mice. *Hum. Exp. Toxicol.* **33**, 185-195 (2014).
153. Y. O. Ilbey *et al.*, Potential chemoprotective effect of melatonin in cyclophosphamide- and cisplatin-induced testicular damage in rats. *Fertil. Steril.* **92**, 1124-1132 (2009).
154. Y. Yang *et al.*, [Effects. of N-acetylcysteine on fluoride-induced endoplasmic reticulum stress in sertoli cells]. *Wei Sheng Yan Jiu* **43**, 805-808, 813 (2014).
155. K. Narayana, S. Verghese, S. S. Jacob, l-Ascorbic acid partially protects two cycles of cisplatin chemotherapy-induced testis damage and oligo-astheno-teratospermia in a mouse model. *Exp. Toxicol. Pathol.* **61**, 553-563 (2009).
156. N. Kilarkaje, A. M. Mousa, M. M. Al-Bader, K. M. Khan, Antioxidants enhance the recovery of three cycles of bleomycin, etoposide, and cisplatin-induced testicular dysfunction, pituitary-testicular axis, and fertility in rats. *Fertil. Steril.* **100**, 1151-1159 (2013).
157. U. B. Das, M. Mallick, J. M. Debnath, D. Ghosh, Protective effect of ascorbic acid on cyclophosphamide- induced testicular gametogenic and androgenic disorders in male rats. *Asian J. Androl.* **4**, 201-207 (2002).
158. T. B. Papa *et al.*, Astaxanthin diferulate as a bifunctional antioxidant. *Free Radic. Res.* **49**, 102-111 (2015).
159. S. Goto *et al.*, Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim. Biophys. Acta* **1512**, 251-258 (2001).
160. Y. M. Naguib, Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* **48**, 1150-1154 (2000).
161. W. Stahl, H. Sies, Lycopene: a biologically important carotenoid for humans? *Arch. Biochem. Biophys.* **336**, 1-9 (1996).
162. M. Viuda-Martos *et al.*, Tomato and tomato byproducts. Human health benefits of lycopene and its application to meat products: a review. *Crit. Rev. Food Sci. Nutr.* **54**, 1032-1049 (2014).
163. A. V. Rao, S. Agarwal, Role of antioxidant lycopene in cancer and heart disease. *J. Am. Coll. Nutr.* **19**, 563-569 (2000).
164. C. C. Liu *et al.*, Lycopene supplementation attenuated xanthine oxidase and myeloperoxidase activities in skeletal muscle tissues of rats after exhaustive exercise. *Br. J. Nutr.* **94**, 595-601 (2005).
165. S. Agarwal, A. V. Rao, Tomato lycopene and its role in human health and chronic diseases. *CMAJ* **163**, 739-744 (2000).
166. N. Sharma, U. C. Goswami, Functioning of Lycopene in Mammalian System: A Review. *Proc Zool Soc* **64**, 1-7 (2011).
167. M. A. Azuine, U. C. Goswami, J. J. Kayal, S. V. Bhide, Antimutagenic and anticarcinogenic effects of carotenoids and dietary palm oil. *Nutr. Cancer* **17**, 287-295 (1992).
168. N. P. Gupta, R. Kumar, Lycopene therapy in idiopathic male infertility--a preliminary report. *Int. Urol. Nephrol.* **34**, 369-372 (2002).

169. M. Matsumoto *et al.*, An antioxidant, N,N'-diphenyl-p-phenylenediamine (DPPD), affects labor and delivery in rats: a 28-day repeated dose test and reproduction/developmental toxicity test. *Food Chem. Toxicol.* **56**, 290-296 (2013).
170. A. B. Roberts, H. F. Deluca, The effect of DPPD on the decarboxylation of retinoic acid in vitro and in vivo. *Arch. Biochem. Biophys.* **129**, 290-295 (1969).
171. A. Galano, M. Francisco Marquez, A. Perez-Gonzalez, Ellagic acid: an unusually versatile protector against oxidative stress. *Chem. Res. Toxicol.* **27**, 904-918 (2014).
172. W. Kalt, C. F. Forney, A. Martin, R. L. Prior, Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* **47**, 4638-4644 (1999).
173. F. Festa *et al.*, Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by the comet assay. *Anticancer Res.* **21**, 3903-3908 (2001).
174. Z. Qiu *et al.*, In vitro antioxidant and antiproliferative effects of ellagic acid and its colonic metabolite, urolithins, on human bladder cancer T24 cells. *Food Chem. Toxicol.* **59**, 428-437 (2013).
175. A. Goraca *et al.*, Lipoic acid - biological activity and therapeutic potential. *Pharmacol. Rep.* **63**, 849-858 (2011).
176. L. Packer, E. H. Witt, H. J. Tritschler, alpha-Lipoic acid as a biological antioxidant. *Free Radic. Biol. Med.* **19**, 227-250 (1995).
177. E. Miadokova, V. Vlckova, V. Duhova, Antimutagenic effect of alpha-lipoic acid on three model test systems. *Pharmazie* **55**, 862-863 (2000).
178. A. Bast, G. R. Haenen, Lipoic acid: a multifunctional antioxidant. *Biofactors* **17**, 207-213 (2003).
179. R. J. Reiter *et al.*, Melatonin in edible plants (phytomelatonin): Identification, concentrations, bioavailability and proposed functions. *World Rev. Nutr. Diet.* **97**, 211-230 (2007).
180. A. R. Tilden, M. A. Becker, L. L. Amma, J. Arciniega, A. K. McGaw, Melatonin production in an aerobic photosynthetic bacterium: an evolutionarily early association with darkness. *J. Pineal Res.* **22**, 102-106 (1997).
181. R. Hardeland *et al.*, Melatonin--a pleiotropic, orchestrating regulator molecule. *Prog. Neurobiol.* **93**, 350-384 (2011).
182. M. Gholami, G. Saki, M. Hemadi, A. Khodadadi, J. Mohammadi-Asl, Melatonin improves spermatogonial stem cells transplantation efficiency in azoospermic mice. *Iran J Basic Med Sci* **17**, 93-99 (2014).
183. C. S. Rocha, L. Rato, A. D. Martins, M. G. Alves, P. F. Oliveira, Melatonin and male reproductive health: relevance of darkness and antioxidant properties. *Curr. Mol. Med.*, (2015).
184. C. Rodriguez *et al.*, Regulation of antioxidant enzymes: a significant role for melatonin. *J. Pineal Res.* **36**, 1-9 (2004).
185. C. M. da Silva *et al.*, Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. *J. Pineal Res.* **51**, 172-179 (2011).
186. F. Radogna *et al.*, Melatonin as an apoptosis antagonist. *Ann. N. Y. Acad. Sci.* **1090**, 226-233 (2006).
187. I. Bejarano *et al.*, Exogenous melatonin supplementation prevents oxidative stress-evoked DNA damage in human spermatozoa. *J. Pineal Res.* **57**, 333-339 (2014).
188. S. S. du Plessis, K. Hagenaar, F. Lampiao, The in vitro effects of melatonin on human sperm function and its scavenging activities on NO and ROS. *Andrologia* **42**, 112-116 (2010).
189. E. A. McClure, C. D. Gipson, R. J. Malcolm, P. W. Kalivas, K. M. Gray, Potential role of N-acetylcysteine in the management of substance use disorders. *CNS drugs* **28**, 95-106 (2014).
190. B. Olsson, M. Johansson, J. Gabrielsson, P. Bolme, Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. *Eur. J. Clin. Pharmacol.* **34**, 77-82 (1988).

191. A. M. Sadowska, Y. K. B. Manuel, W. A. De Backer, Antioxidant and anti-inflammatory efficacy of NAC in the treatment of COPD: discordant in vitro and in vivo dose-effects: a review. *Pulm. Pharmacol. Ther.* **20**, 9-22 (2007).
192. N. De Vries, S. De Flora, N-acetyl-l-cysteine. *J. Cell. Biochem. Suppl.* **17F**, 270-277 (1993).
193. K. Erkkila, V. Hirvonen, E. Wuokko, M. Parvinen, L. Dunkel, N-acetyl-L-cysteine inhibits apoptosis in human male germ cells in vitro. *J. Clin. Endocrinol. Metab.* **83**, 2523-2531 (1998).
194. E. M. Perchellet, E. A. Maatta, N. L. Abney, J. P. Perchellet, Effects of diverse intracellular thiol delivery agents on glutathione peroxidase activity, the ratio of reduced/oxidized glutathione, and ornithine decarboxylase induction in isolated mouse epidermal cells treated with 12-O-tetradecanoylphorbol-13-acetate. *J. Cell. Physiol.* **131**, 64-73 (1987).
195. L. Bonanomi, A. Gazzaniga, Toxicological, pharmacokinetic and metabolic studies on acetylcysteine. *Eur. J. Respir. Dis. Suppl.* **111**, 45-51 (1980).
196. R. E. Johnston, H. C. Hawkins, J. H. Weikel, Jr., The toxicity of N-acetylcysteine in laboratory animals. *Semin. Oncol.* **10**, 17-24 (1983).
197. F. Wang *et al.*, Protective effects of -acetylcysteine on cisplatin-induced oxidative stress and DNA damage in HepG2 cells. *Exp Ther Med* **8**, 1939-1945 (2014).
198. K. Shimamoto *et al.*, Antioxidant N-acetyl-L-cysteine (NAC) supplementation reduces reactive oxygen species (ROS)-mediated hepatocellular tumor promotion of indole-3-carbinol (I3C) in rats. *J. Toxicol. Sci.* **36**, 775-786 (2011).
199. L. C. De la Cruz Rodriguez, C. R. Araujo, S. E. Posleman, M. R. Rey, Attenuation of gentamicin-induced nephrotoxicity: trimetazidine versus N-acetyl cysteine. *J. Appl. Toxicol.* **30**, 343-353 (2010).
200. J. Mandl, A. Szarka, G. Banhegyi, Vitamin C: update on physiology and pharmacology. *Br. J. Pharmacol.* **157**, 1097-1110 (2009).
201. A. Sorice *et al.*, Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini Rev. Med. Chem.* **14**, 444-452 (2014).
202. M. J. O'Neil, *The Merck index : an encyclopedia of chemicals, drugs, and biologicals*. (Merck, Whitehouse Station, N.J., ed. 14th, 2006).
203. E. R. Monsen, Dietary reference intakes for the antioxidant nutrients: vitamin C, vitamin E, selenium, and carotenoids. *J. Am. Diet. Assoc.* **100**, 637-640 (2000).
204. H. Fukumura *et al.*, Effect of ascorbic acid on reactive oxygen species production in chemotherapy and hyperthermia in prostate cancer cells. *J. Physiol. Sci.* **62**, 251-257 (2012).
205. S. Silici, O. Ekmekcioglu, G. Eraslan, A. Demirtas, Antioxidative effect of royal jelly in cisplatin-induced testes damage. *Urology* **74**, 545-551 (2009).
206. X. Li, C. Huang, Y. Xue, Contribution of lipids in honeybee (Apis mellifera) royal jelly to health. *J. Med. Food* **16**, 96-102 (2013).
207. N. Liang, D. D. Kitts, Antioxidant Property of Coffee Components: Assessment of Methods that Define Mechanisms of Action. *Molecules* **19**, 19180-19208 (2014).
208. V. Fogliano, F. J. Morales, Estimation of dietary intake of melanoidins from coffee and bread. *Food Funct* **2**, 117-123 (2011).
209. F. Natella, M. Nardini, I. Giannetti, C. Dattilo, C. Scaccini, Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* **50**, 6211-6216 (2002).
210. T. R. Dias *et al.*, Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. *Toxicology* **328**, 12-20 (2015).
211. M. Khazaei *et al.*, Protective effects of subchronic caffeine administration on cisplatin induced urogenital toxicity in male mice. *Indian J. Exp. Biol.* **50**, 638-644 (2012).
212. H. Mukhtar, Z. Y. Wang, S. K. Katiyar, R. Agarwal, Tea components: antimutagenic and anticarcinogenic effects. *Prev. Med.* **21**, 351-360 (1992).

213. N. P. Seeram *et al.*, Catechin and caffeine content of green tea dietary supplements and correlation with antioxidant capacity. *J. Agric. Food Chem.* **54**, 1599-1603 (2006).
214. A. D. Martins *et al.*, Effect of white tea (Camellia sinensis (L.)) extract in the glycolytic profile of Sertoli cell. *Eur. J. Nutr.* **53**, 1383-1391 (2014).
215. T. R. Dias *et al.*, White tea as a promising antioxidant medium additive for sperm storage at room temperature: a comparative study with green tea. *J. Agric. Food Chem.* **62**, 608-617 (2014).
216. A. Masala *et al.*, Use of testosterone to prevent cyclophosphamide-induced azoospermia. *Ann. Intern. Med.* **126**, 292-295 (1997).
217. A. Cigni *et al.*, Hormonal strategies for fertility preservation in patients receiving cyclophosphamide to treat glomerulonephritis: a nonrandomized trial and review of the literature. *Am. J. Kidney Dis.* **52**, 887-896 (2008).
218. M. L. Meistrich, G. Shetty, Hormonal suppression for fertility preservation in males and females. *Reproduction* **136**, 691-701 (2008).
219. L. M. Pogach, Y. Lee, S. Gould, W. Giglio, H. F. Huang, Partial prevention of procarbazine induced germinal cell aplasia in rats by sequential GnRH antagonist and testosterone administration. *Cancer Res.* **48**, 4354-4360 (1988).
220. L. M. Glode, J. M. Shannon, N. Malik, T. Nett, Protection of rat spermatogenic epithelium from damage induced by procarbazine chemotherapy. *Br. J. Cancer* **62**, 61-64 (1990).
221. M. L. Meistrich, N. Parchuri, G. Wilson, B. Kurdoglu, M. Kangasniemi, Hormonal protection from cyclophosphamide-induced inactivation of rat stem spermatogonia. *J. Androl.* **16**, 334-341 (1995).
222. B. Kurdoglu, G. Wilson, N. Parchuri, W. S. Ye, M. L. Meistrich, Protection from radiation-induced damage to spermatogenesis by hormone treatment. *Radiat. Res.* **139**, 97-102 (1994).
223. A. B. Thomson *et al.*, Investigation of suppression of the hypothalamic-pituitary-gonadal axis to restore spermatogenesis in azoospermic men treated for childhood cancer. *Hum. Reprod.* **17**, 1715-1723 (2002).
224. J. H. Waxman *et al.*, Failure to preserve fertility in patients with Hodgkin's disease. *Cancer Chemother. Pharmacol.* **19**, 159-162 (1987).
225. E. D. Kreuser, W. D. Hetzel, R. Hautmann, E. F. Pfeiffer, Reproductive toxicity with and without LHRHA administration during adjuvant chemotherapy in patients with germ cell tumors. *Horm. Metab. Res.* **22**, 494-498 (1990).
226. B. Sredni, M. Albeck, G. Kazimirsky, F. Shalit, The immunomodulator AS101 administered orally as a chemoprotective and radioprotective agent. *Int. J. Immunopharmacol.* **14**, 613-619 (1992).
227. B. Sredni *et al.*, The protective role of the immunomodulator AS101 against chemotherapy-induced alopecia studies on human and animal models. *Int. J. Cancer* **65**, 97-103 (1996).
228. G. Halpert, B. Sredni, The effect of the novel tellurium compound AS101 on autoimmune diseases. *Autoimmun. Rev.* **13**, 1230-1235 (2014).
229. Y. Kalechman *et al.*, Protective and restorative role of AS101 in combination with chemotherapy. *Cancer Res.* **51**, 1499-1503 (1991).
230. A. Carmely *et al.*, Protective effect of the immunomodulator AS101 against cyclophosphamide-induced testicular damage in mice. *Hum. Reprod.* **24**, 1322-1329 (2009).
231. O. Nicolatou-Galitis *et al.*, Systematic review of amifostine for the management of oral mucositis in cancer patients. *Support. Care Cancer* **21**, 357-364 (2013).
232. J. C. Junn *et al.*, The effect of amifostine on submandibular gland histology after radiation. *Int J Otolaryngol* **2012**, 508279 (2012).
233. L. C. Lirdi, T. Stumpp, E. Sasso-Cerri, S. M. Miraglia, Amifostine protective effect on cisplatin-treated rat testis. *Anat Rec (Hoboken)* **291**, 797-808 (2008).

234. V. Vendramini, E. Sasso-Cerri, S. M. Miraglia, Amifostine reduces the seminiferous epithelium damage in doxorubicin-treated prepubertal rats without improving the fertility status. *Reprod. Biol. Endocrinol.* **8**, 3 (2010).
235. L. R. Schover, L. A. Rybicki, B. A. Martin, K. A. Bringelsen, Having children after cancer. A pilot survey of survivors' attitudes and experiences. *Cancer* **86**, 697-709 (1999).
236. K. Boekelheide, Mechanisms of toxic damage to spermatogenesis. *J. Natl. Cancer Inst. Monogr.*, 6-8 (2005).
237. S. Joel, The clinical pharmacology of etoposide: an update. *Cancer Treat. Rev.* **22**, 179-221 (1996).
238. J. F. Bishop, Etoposide in the treatment of leukemias. *Semin. Oncol.* **19**, 33-38 (1992).
239. S. Harel, C. Ferme, C. Poirot, Management of fertility in patients treated for Hodgkin's lymphoma. *Haematologica* **96**, 1692-1699 (2011).
240. F. K. Okada, T. Stumpp, S. M. Miraglia, Carnitine reduces testicular damage in rats treated with etoposide in the prepubertal phase. *Cell Tissue Res.* **337**, 269-280 (2009).
241. A. Rabaça, M. Sousa, M. G. Alves, P. F. Oliveira, R. Sá, Novel drug therapies for fertility preservation in men undergoing chemotherapy: clinical relevance of protector agents. *Curr. Med. Chem.*, (2015). (in print)
242. H. Ciftci, A. Verit, M. Savas, E. Yeni, O. Erel, Effects of N-acetylcysteine on semen parameters and oxidative/antioxidant status. *Urology* **74**, 73-76 (2009).
243. World Health Organization (WHO), *WHO laboratory manual for the examination and processing of human semen*. Examination and processing of human semen (WHO Press, Geneva, Switzerland ed. 5th, 2010), pp. 286.
244. R. Sá, M. Cunha, E. Rocha, A. Barros, M. Sousa, Sperm DNA fragmentation is related to sperm morphological staining patterns. *Reprod. Biomed. Online*, (2015). (epub ahead of print)
245. R. L. Levine *et al.*, Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **186**, 464-478 (1990).
246. M. G. Alves, A. Neuhaus-Oliveira, P. I. Moreira, S. Socorro, P. F. Oliveira, Exposure to 2,4-dichlorophenoxyacetic acid alters glucose metabolism in immature rat Sertoli cells. *Reprod. Toxicol.* **38**, 81-88 (2013).
247. J. F. Griveau, D. Le Lannou, Reactive oxygen species and human spermatozoa: physiology and pathology. *Int. J. Androl.* **20**, 61-69 (1997).
248. E. de Lamirande, C. Gagnon, Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *J. Androl.* **13**, 368-378 (1992).
249. T. R. Dias, M. G. Alves, B. M. Silva, P. F. Oliveira, Sperm glucose transport and metabolism in diabetic individuals. *Mol. Cell. Endocrinol.* **396**, 37-45 (2014).
250. A. C. Williams, W. C. Ford, The role of glucose in supporting motility and capacitation in human spermatozoa. *J. Androl.* **22**, 680-695 (2001).
251. T. H. Hereng *et al.*, Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. *Hum. Reprod.* **26**, 3249-3263 (2011).
252. R. W. Hanson, F. J. Ballard, The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. *Biochem. J.* **105**, 529-536 (1967).
253. L. Lazaros *et al.*, Phosphatidylethanolamine N-methyltransferase and choline dehydrogenase gene polymorphisms are associated with human sperm concentration. *Asian J. Androl.* **14**, 778-783 (2012).
254. A. R. Johnson *et al.*, Deletion of murine choline dehydrogenase results in diminished sperm motility. *FASEB J.* **24**, 2752-2761 (2010).

255. M. Sieniawski *et al.*, Assessment of male fertility in patients with Hodgkin's lymphoma treated in the German Hodgkin Study Group (GHSG) clinical trials. *Ann. Oncol.* **19**, 1795-1801 (2008).
256. J. Sagara, S. Bannai, N. Shikano, N. Makino, Conflicting effects of N-acetylcysteine on purified neurons derived from rat cortical culture. *Neuroreport* **21**, 416-421 (2010).
257. B. Lohrke *et al.*, N-acetylcysteine impairs survival of luteal cells through mitochondrial dysfunction. *Cytometry A* **77**, 310-320 (2010).
258. D. Sakkas, J. G. Alvarez, Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil. Steril.* **93**, 1027-1036 (2010).
259. D. Sakkas *et al.*, Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum. Reprod.* **13 Suppl 4**, 11-19 (1998).
260. F. Marchetti, F. S. Pearson, J. B. Bishop, A. J. Wyrobek, Etoposide induces chromosomal abnormalities in mouse spermatocytes and stem cell spermatogonia. *Hum. Reprod.* **21**, 888-895 (2006).
261. S. Matulis, M. A. Handel, Spermatocyte responses in vitro to induced DNA damage. *Mol. Reprod. Dev.* **73**, 1061-1072 (2006).
262. L. B. Russell, P. R. Hunsicker, D. K. Johnson, M. D. Shelby, Unlike other chemicals, etoposide (a topoisomerase-II inhibitor) produces peak mutagenicity in primary spermatocytes of the mouse. *Mutat. Res.* **400**, 279-286 (1998).
263. S. De Flora *et al.*, Chemopreventive properties and mechanisms of N-Acetylcysteine. The experimental background. *J. Cell. Biochem. Suppl.* **22**, 33-41 (1995).
264. S. De Flora, F. D'Agostini, A. Izzotti, R. Balansky, Prevention by N-acetylcysteine of benzo[a]pyrene clastogenicity and DNA adducts in rats. *Mutat. Res.* **250**, 87-93 (1991).
265. S. De Flora, A. Izzotti, F. D'Agostini, R. M. Balansky, Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. *Carcinogenesis* **22**, 999-1013 (2001).
266. S. Lopes, A. Jurisicova, J. G. Sun, R. F. Casper, Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Hum. Reprod.* **13**, 896-900 (1998).
267. A. K. Talley *et al.*, Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: protection by the antioxidant N-acetylcysteine and the genes bcl-2 and crmA. *Mol. Cell. Biol.* **15**, 2359-2366 (1995).
268. J. T. Wolfe, D. Ross, G. M. Cohen, A role for metals and free radicals in the induction of apoptosis in thymocytes. *FEBS Lett.* **352**, 58-62 (1994).
269. B. K. Sinha, M. A. Trush, B. Kalyanaraman, Free radical metabolism of VP-16 and inhibition of anthracycline-induced lipid peroxidation. *Biochem. Pharmacol.* **32**, 3495-3498 (1983).
270. T. Oeda, R. Henkel, H. Ohmori, W. B. Schill, Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? *Andrologia* **29**, 125-131 (1997).
271. M. A. Abdelmegeed, S. Jang, A. Banerjee, J. P. Hardwick, B. J. Song, Robust protein nitration contributes to acetaminophen-induced mitochondrial dysfunction and acute liver injury. *Free Radic. Biol. Med.* **60**, 211-222 (2013).
272. Y. Shao, C. C. Ling, X. Q. Liu, High concentrations of glucose suppress etoposide-induced cell death of B-cell lymphoma through BCL-6. *Biochem. Biophys. Res. Commun.* **450**, 227-233 (2014).
273. A. K. Palo, P. Sahu, R. C. Choudhury, Etoposide-induced cytogenotoxicity in mouse spermatogonia and its potential transmission. *J. Appl. Toxicol.* **25**, 94-100 (2005).
274. L. Gandini *et al.*, Study of apoptotic DNA fragmentation in human spermatozoa. *Hum. Reprod.* **15**, 830-839 (2000).

275. J. D. Lee, Y. Kamiguchi, R. Yanagimachi, Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphologies after injection into mouse oocytes. *Hum. Reprod.* **11**, 1942-1946 (1996).
276. J. P. Dadoune, M. J. Mayaux, M. L. Guihard-Moscato, Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. *Andrologia* **20**, 211-217 (1988).
277. R. H. Martin *et al.*, A comparison of the frequency of sperm chromosome abnormalities in men with mild, moderate, and severe oligozoospermia. *Biol. Reprod.* **69**, 535-539 (2003).
278. K. Nowicka-Bauer, M. Kurpisz, Current knowledge of the human sperm proteome. *Expert Rev. Proteomics* **10**, 591-605 (2013).

VII. Annexes

Annex I: Material and Methods
Annex II: NAC concentration
Annex III: Semen parameters reference values
Annex IV: Spermatozoa protein extraction

I. Material and Methods

1. Ethical considerations

Ethical guidelines were followed in the conduct of research, with written informed consent having been obtained before the beginning of work. This work did not involve human or animal experiments. An approval by an Ethics Committee and the Declaration of Helsinki as revised in Tokyo 2004 on human experimentation does not apply to this kind of work. Thus, according to the standards of National Law (PMA, Law 32/2006) and Council (CNPMA, 2008) criteria on Medically Assisted Procreation. Human semen samples were obtained from patients performing spermiogram analysis at the private clinic Centro de Genética da Reprodução Prof. Alberto Barros (CGR-ABarros) after they had given informed and written consent.

2. Patient and sample selection

The semen samples used in this study were acquired from 10 individuals that went for semen analysis at CGR between October and November 2014. In order to be selected for this study, samples had to meet the following inclusion criteria:

- No known diseases;
- No prolonged pharmacological therapy;
- Normal physical exam ;
- Normal karyotype, with no micro deletions of the Y chromosome;
- Normal hormonal profile;
- Semen analysis presenting no agglutination, immature forms, leukocytes or microorganisms and a sperm concentration ≥ $15x10^6$/ml.

3. Experimental design

In this study, each semen sample selected by the above criteria was divided into four experimental groups subjected to different conditions (Figure VII-1). After removal of the seminal fluid semen parameters were evaluated at CGR and a portion from samples meeting our criteria was transferred for research. Each sample was diluted in sperm preparation medium (SPM; Origio, Jyllinge, Denmark), supplemented or not with etoposide

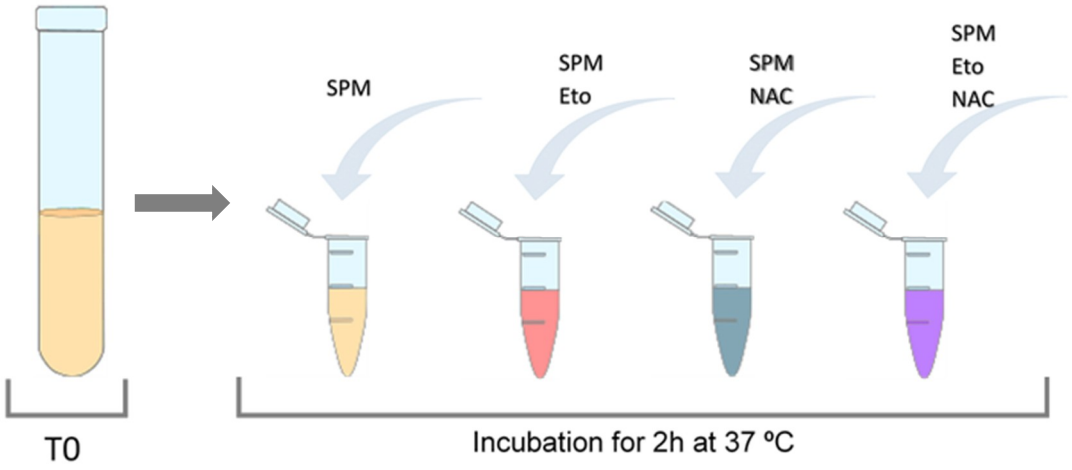

Figure VII-1. Schematic representation of the experimental groups. Each sample was analysed at the end of the incubation period. Samples were incubated in four different conditions. SPM corresponded to the control group and the three remaining groups were of etoposide, NAC, or both agents in combination.

(Sigma, St. Louis, MO, USA), NAC (Sigma) or both agents in combination, and incubated for a 2h period, in a humidified atmosphere of air containing 5% CO_2 at 37ºC. Several NAC concentrations were evaluated and analysed in order to maximize results without damaging the spermatozoa (Annexes II). Each sample was divided into the following experimental conditions:

- Control group (CT group): samples were incubated in SPM containing a 0.05% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) concentration;
- Etoposide group (Eto group): samples were incubated in SPM with 0.05% DMSO in combination with a pharmacologically relevant concentration of etoposide (25 µg/ml);
- N-acetylcysteine group (NAC group): samples were incubated in SPM with 0.05% DMSO in combination with 50 µM of NAC;
- Etoposide and N-acetylcysteine group (Eto+NAC group): samples were incubated in SPM with 0.05% DMSO in combination with 25 µg/ml of etoposide and 50 µM of NAC.

After the incubation period we evaluated spermatozoa motility, hipoosmolarity, morphology, DNA fragmentation and chromatin condensation. The remaining sample volume was then centrifuged at 670 Xg and both supernatant and pellet stored separately

at -20°C for oxidative damage analysis and for extracellular metabolites determination, respectively.

4. Semen analysis (Spermiogram)

Semen samples were obtained by masturbation into sterile plastic containers after a 3 to 5 day period of sexual abstinence. Immediately after collection, samples were placed at 37 °C while the semen liquefied. The macroscopic examination, which includes liquefaction, viscosity, appearance, volume and pH, as well as the microscopic examination were performed according to WHO guidelines 2010 (*243*). The normal value range for sample parameters and protocols for the macroscopic exam can be found in Annexes III and IV, respectively.

4.1. Macroscopic examination

4.1.1. Liquefaction

Immediately after ejaculation into the sterilized container, semen is typically a semi-solid coagulated mass. Within a few minutes at room temperature (RT), or in a CO_2 incubator at 37ºC, semen regularly begins to liquefy. When the semen sample is liquefied it becomes homogeneous and watery. The complete sample, if normal, generally liquefies within 15 to 30 min at RT, not taking longer than an hour.

4.1.2. Viscosity

After liquefaction, the viscosity of the sample is evaluated by gentle aspiration using a pipette, allowing sperm to fall by gravity and observing the length of the thread. Viscosity is considered normal when the semen lefts the pipette into small droplets. If the sample dropped in threads of more than 2 cm long it is considered abnormal. High viscosity may interfere with the evaluation of sperm parameters such as motility and concentration.

4.1.3. Appearance of the ejaculate

Samples appearance at RT is observed after the liquefaction process is complete. Samples are considered normal when the liquefied semen has a

homogeneous, gray-opalescent appearance. Samples may however look less opaque when sperm concentration is very low. Color may also be different. Samples with a red-brown color indicate the presence of red blood cells in the ejaculate (haemospermia), and yellow samples are characteristic of man with jaundice, taking certain vitamins or drugs, or the presence of infections.

4.1.4. Smell

Normal semen samples have a characteristic odor regarded as *sui generis*. Abnormal samples may not have smell or present a displeasing fetid smell.

4.1.5. Volume

Total volume of ejaculate is mostly constituted by fluids from the seminal vesicles and prostate gland, in addition to a small amount from the bulbourethral glands and the epididymis. The volume is directly measured using a graduated device. Low semen volume is characteristic of obstruction of the ejaculatory duct, congenital bilateral absence of the vas deferens, sample collection problems with loss of a fraction of the ejaculate, partial retrograde ejaculation or androgen deficiency. High volumes point to exudation in cases of active inflammation of the accessory glands.

4.1.6. pH

Semen pH reveals the balance between the alkaline secretion of seminal vesicles (fructose, citrate, inositol, prostaglandins) and the acidic secretion of the prostate gland (citric acid, acid phosphatase, amylase and fibrolisine). The pH is measured after liquefaction and a sample is considered normal when the pH value is at least 7.2 (lower threshold value).

4.2. Microscopic examination

4.2.1. Sperm concentration

Concentration is determined in Neubauer chamber after sample fixation with a solution that incites an osmotic shock. The lower reference limit for spermatozoa concentration is $15x10^6$/ ml (*243*). After, samples sre diluted in distilled H_2O

according WHO recommendations (Table VII-1), they are then added to a Neubauer chamber, placed in a humidified container for 10 to 15 min and counted.

Table VII-1 | Sperm sample dilution values.

Number spermatozoa / microscopic field (400X)	Dilution factor	Semen (µl)	Distilled H_2O (µl)
<3	1:2	50	50
3-10	1:5	50	200
10-25	1:10	50	450
25-100	1:20	50	950
>100	1:50	50	2450

4.2.2. Sperm motility

Sperm motility should be assessed as soon as possible after sample liquefaction and is closely related to male fertility and pregnancy rates (*243*).

Sperm motility is evaluated and graded, differentiating spermatozoa with progressive or non-progressive motility from those that are immotile, as follows:

- Progressive motility (PR): spermatozoa actively moving independently of the speed.
- Non-progressive motility (NP): all other patterns of motility with an absence of progression, e.g. when only a flagella beat can be observed.
- Immobility (IM): absent of movement.

To evaluate sperm motility, semen samples are carefully homogenised and wet preparations are made onto slides previously heated on a stage warmer to a temperature of 37°C. Slides are then examined with an optical microscope at a total magnification of 400x. Motility is evaluated for at least 200 spermatozoa with the aid of a laboratory counter (Digisystem Laboratory Instruments Inc., Taipei, China). In each field PR spermatozoa are initially counted, then NP and finally IM spermatozoa. Results are then expressed in the percentage of each category per slide.

4.2.3. Sperm Vitality: Hypo-osmotic assay

Sperm vitality is estimated by assessing the membrane integrity of the spermatozoa. Sperm vitality can be assessed by evaluating the integrity of the flagellum. The hypo-osmotic swelling (HOS) test presumes that only live cells, those with intact membranes, will swell when incubated in hypotonic solutions. Therefore, live cells are distinguishable by the swelling of the sperm tail. Swollen spermatozoa are identified by changes in the shape of the cell, as indicated by coiling of the tail (Figure VII-2). The HOS test is considered within the normal range when the percentage of reactive spermatozoa is above 58% (*243*).

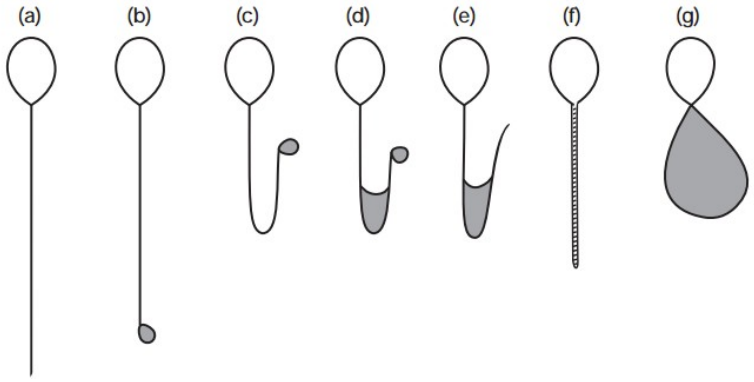

Figure VII-2. Hypo-osmotic assay. Schematic representation of usual morphological changes in human spermatozoa subjected to hypo-osmotic swelling test. Spermatozoon (a) represents a dead spermatozoa with no reaction of the tail. Spermatozoa (b) to (g) represent vital cell with coiling of the tail, represented as grey areas. Adapted from WHO, 2010.

To evaluate sperm vitality through the HOS assay, 10 µl of each semen sample are incubated at 37°C for 30 minutes in 100 µl of the swelling solution (0.735 g of sodium citrate di-hydrate (Merck) with 1.351 g of D-fructose (Sigma) in 100 ml of purified water). A 10 µl aliquot of the sample is then transferred onto a clean slide, cover with a coverslip and examined with an optic microscope at a 400X magnification (Figure VII-3). Two hundred spermatozoa are evaluated as un-swollen (dead) or swollen (vital) and results are presented as percentages.

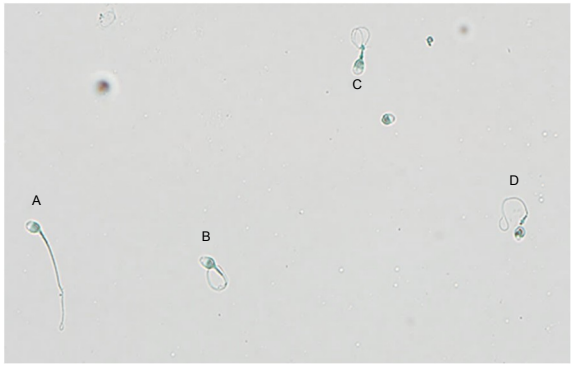

Figure VII-3. Photo of human spermatozoa subjected to hypo-osmotic swelling test. When normal spermatozoa are incubated with a hypotonic solution the tail swells, curling on itself. This phenomena can be observed in an optic microscope. Spermatozoa A to D represent live spermatozoa with different forms of tail coiling. Total magnification: ×400.

4.2.4. Sperm morphology

Assessment of sperm morphology is associated with various complications related to lack of objectivity, variable morphology, variation in interpretation or poor performance in external quality-control assessments. For a spermatozoon to be considered normal, both its head and tail must be normal. All borderline forms should be considered abnormal. Human semen samples contain spermatozoa with different kinds of malformations. A higher percentage of abnormal spermatozoa is characteristic of a defective spermatogenesis and some epididymal pathologies, and is normally a sign of decreased fertilizing potential. Morphological defects have also been associated with increased DNA fragmentation (*274*), an increased incidence of structural chromosomal aberrations (*275*), immature chromatin condensation (*276*) and aneuploidy (*277*).

The following categories of spermatozoa morphological defects should be noted (Figure VII-4):

- Head defects (HD): large or small, tapered, pyriform, round, amorphous, vacuolated, small or large acrosome areas, double heads, or any combination of these.
- Neck and mid-piece defects (ND): asymmetrical insertion of the mid-piece into the head, thick or irregular, sharply bent, abnormally thin, or any combination of these.
- Tail defects (TD): short, multiple, broken, smooth hairpin bends, sharply angulated bends, of irregular width, coiled, or any combination of these.
- Excess residual cytoplasm (ERC): this is associated with abnormal spermatozoa produced from a defective spermatogenesis. .

Staining procedure:

1. A smear for each sample is prepared on a slide and air-dried;
2. The smear is then fixated with 96% ethanol (Merck) for 15 min at RT;
3. After fixation samples are stained as follows:
    i. Ethanol 50% for 2 to 3 min;
    ii. 10 immersion in purified H_2O;
    iii. Harris's haematoxylin (Merck) for 8 min;
    iv. Running tap H_2O for 5 min;
    v. 4 to 8 immersions in 37% acidic ethanol (1ml of hydrochloric acid (Merck) at in 100 ml of ethanol and 100 ml of distilled H_2O);
    vi. Running tap H_2O for 5 min;
    vii. 1 immersion in purified H_2O;
    viii. 10 immersions for each increasing concentration of ethanol (50, 70, 80 and 96%);
    ix. Orange G-6 (Merck) for 4 min;
    x. 10 immersions in 96% ethanol (2x);
    xi. EA-50 (Sigma) for 5 min;
    xii. 10 immersions in 96% ethanol (3x)
4. After the staining process is completed, slides are observed in the optical microscope with the immersion objective 100x (1000x total magnification);
5. 200 spermatozoa are counted with the aid of a laboratory counter.
6. Spermatozoa are classified as normal or abnormal, with different defects of the head, mid-piece, tail or cytoplasmic excess).
7. Results are expressed as the percentage of spermatozoa in each category.

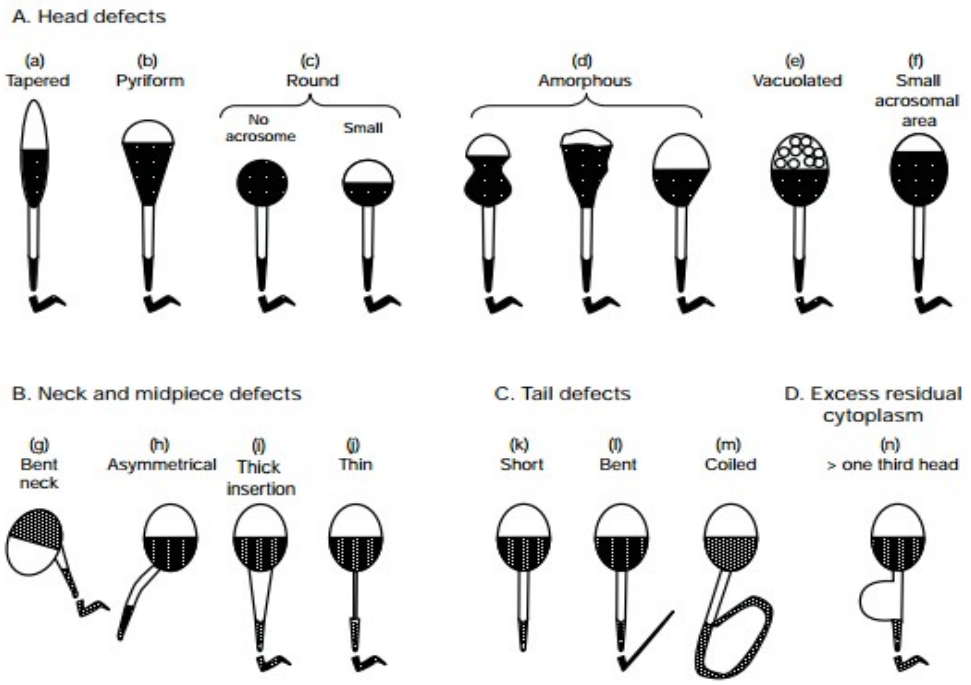

Figure VII-4. Schematic representation of abnormal forms of human spermatozoa. Human semen samples can present a significant number of spermatozoa with head, neck and tail defects, as well as excess of residual cytoplasm. Adapted from WHO, 2010.

5. Sperm DNA integrity Analysis

Several methods have been used to test the normality of sperm chromatin and DNA. Methods include those based on dyes that bind to histones (aniline blue) and assessment of DNA strand breaks, such as terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-nick-end labelling (TUNEL), respectively.

5.1. Determination of chromatin condensation

Sperm chromatin condensation is assessed by acidic aniline blue staining (AB; Merck). Sperm smears are prepared onto adhesive microscope slides and fixed in 3% glutaraldehyde (Merck) in 0.2M PBS (potassium phosphate, sodium chloride, sodium phosphate; Merck) solution for 30 min at RT. Slides are then stained with 5% aqueous aniline blue solution mixed with 4% acetic acid pH 3.5 (Merck) for 5 min at RT. After the staining procedure is completed, 200 morphologically normal spermatozoa are evaluated in a bright field microscope (Olympus CX21, Melville, NY) with the immersion objective 100X at a total magnification of 1000X (Figure VII-5). Results are expressed as the percentage of dark blue stained spermatozoa

(indicator of immature histone-rich nuclei). Samples with more than 20% of the spermatozoa showing dark blue staining are considered abnormal.

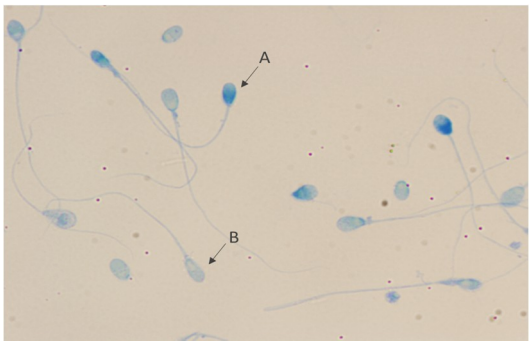

Figure VII-5. Chromatin condensation analyses of human spermatozoa by acid-aniline blue staining. Spermatozoon positive for the acid-aniline blue coloration (A) displays immature chromatin condensation. Spermatozoon negative for the acid-aniline blue coloration (B) presents normal chromatin condensation.

5.2. Determination of sperm DNA fragmentation

For each sample sperm DNA fragmentation is evaluated by the TUNEL assay at the end of each condition, using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Sperm smears are prepared onto adhesive microscope slides and fixed in a 4 % paraformaldehyde (Merck) in PBS solution for 1h at RT. Slides were then washed in PBS and permeabilized with a 0,1 % Triton-X in 0,1 % sodium citrate (Sigma) solution for 2 min at 4 °C. Once again slides were washed in PBS and then incubated for 1 h in a dark humid chamber at 37 ºC with 50 µl of TUNEL mixture. After counterstaining with Vectashield antifade medium containing 4′6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), 200 morphologically normal spermatozoa were evaluated in a Leitz DMRBE fluorescence microscope (Leica, Wetzlar, Germany) (Figure VII-6). The number of permatozoa emitting green fluorescence (TUNEL-positive) was recorded as a percentage of the total number of sperm counted (DAPI stained).

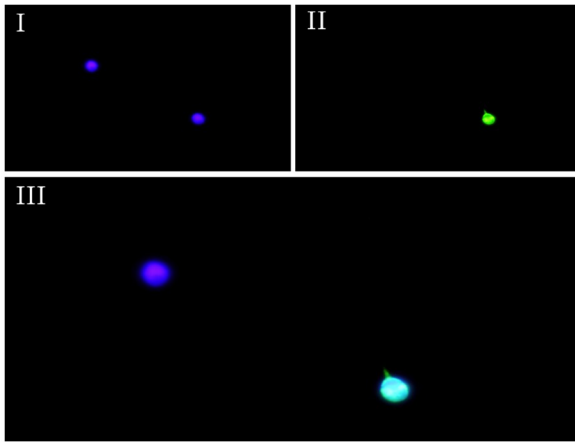

Figure VII-6. DNA fragmentation analyses of human spermatozoa by dUTP nick-end labelling (TUNEL) assay. In image I spermatozoa are stained in blue by DAPI. In image II spermatozoa with fragmented DNA are stained in green with FITC. In image III, the merge of I and II, reveals the fragmented spermatozoa in the total of spermatozoa observed.

6. Sperm protein extraction

To analyse sperm proteome there is a need to identify the proper protein solubilization buffer and protocol. The choice of lysis buffer and experimental procedure is critical for the solubilisation of the sperm proteins (*278*). In order to identify the best protocol for protein extraction of human sperm, eight different procedures were evaluated (Annex IV A) prior to final sample protein extraction. The procedure that presented the best results (Annex IV B) was then employed for protein extraction of the reserved pellets.

7. Colorimetric detection and quantitation of total protein

To quantify sample's protein content after extraction a BCA Protein Assay Kit (Thermo Fisher Scientific) was implemented according to the manufacturer's instructions. This kit was a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total cellular protein content. The quantification is based on a purple-coloured reaction, due to the chelation of two molecules of BCA with one cuprous ion, with strong absorbance at 562nm. The absorbance values have a linear correlation with increasing protein concentrations. Sperm protein concentrations were determined based on a standard curve of known concentrations of bovine serum albumin (BSA, Thermo Fisher Scientific).

8. Sperm oxidative stress analysis

Oxidative stress can be assessed by the cellular protein carbonyl content, a marker for protein oxidation, the measurement of aldehyde products such as 4-hydroxynonenal (4-HNE), an indicator of lipid peroxidation, and by the quantification of 3-nitrotyrosine (3-NT), an indicator of superoxide-dependent peroxynitrite formation. The content of protein carbonyl groups, 4-HNE and 3-NT in spermatozoa from the different experimental groups was evaluated using the Slot-Blot technique and specific antibodies.

8.1 Lipid peroxidation and protein nitration procedure:

i. Protein samples were diluted to a final concentration of 0,03 µg/µl;
ii. PVDF membranes were activated in methanol for 30 seconds and purified water for 5 min;
iii. 100 µl of each sample was added to the Slot-Blot wells;
iv. Samples were towed from the wells with a vacuum pump until wells were dry;

v. The resulting membranes were blocked for 90 min in 5% low fat milk powder dissolved in TBS (Merck) with 0.05% Tween 20 (Sigma);
vi. Membranes were then incubated overnight at 4ºC with a goat anti-4-HNE antibody (1:5000, AB5605, Merck) for lipid peroxidation and with rabbit nitro-tyrosine antibody (1:5000, 9691, Cell Signaling Tecnology, Massachusetts, USA) for protein nitration;
vii. Membranes were incubated with secondary antibodies at for 1h at RT, using rabbit anti-goat IgG-AP (for 4-HNE) (1:5000, A4187, Sigma) or goat anti-rabbit IgG-AP (for 3-NT) (1:5000, sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany);
viii. Membranes were then reacted with ECFTM substrate (GE Healthcare, Buckinghamshire, UK) and read in a Bio-Rad Gel Doc XR+ (Bio-Rad, Hemel Hempstead, UK);
ix. Each band density was quantified using Quantity One Software Version 4.6.9 (Bio-Rad).

8.2 Carbonyl groups analysis procedure:

i. Protein samples were diluted to a final concentration of 0,05 µg/µl;
ii. Protein samples were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain 2,4- dinitrophenyl (DNP) (*245*).
iii. PVDF membranes were activated in methanol for 30 seconds and purified water for 5 min;
iv. 100 µl of each sample was added to the Slot-Blot wells;
v. Samples were towed from the wells with a vacuum pump until wells were dry;
vi. The resulting membranes were blocked for 90 min in 5% low fat milk powder dissolved in TBS-T with 0.05% Tween 20;
vii. The resulting PVDF membranes were incubated overnight at 4ºC with a rabbit anti-DNP (1:5000, D9656, Sigma);
viii. Membranes were incubated with a secondary antibody for 1h at RT, using a goat anti-rabbit IgG-AP (3-NT and DNP) (1:5000, sc-2007, Santa Cruz Biotechnology);
x. Membranes were then reacted with ECFTM substrate (GE Healthcare) and read in a Bio-Rad Gel Doc XR+ (Bio-Rad);
xi. Each band density was quantified using Quantity One Software Version 4.6.9 (Bio-Rad).

9. Proton Nuclear Magnetic Resonance (^{1}H-NMR)

The extracellular metabolites from each sample after the incubation period were acquired by ^{1}H-NMR. Sodium fumarate at a final concentration of 1 mM was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): H1-α glucose (doublet, 5.22); choline (singlet, 3.18); pyruvate (singlet, 2.38); acetate (singlet, 1.9) and lactate (doublet, 1.33 ppm). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn NMR, Livermore, CA, USA).

10. Statistical Analysis

The statistical significance among the experimental groups was assessed by ANOVA, followed by Fisher's LSD. Two-way ANOVA was performed for sperm morphology analysis and one-way ANOVA for all the remaining parameters evaluated. All data are shown as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). All *P values* < 0.05 were considered statistically significant.

II. NAC concentration

NAC is primarily used as a mucolytic agent but is now being tested for the treatment of several health conditions due to its chemopreventive and antioxidant features. However, *in vitro* studies with this agent and human spermatozoa are scarce and there is little information regarding the optimal concentrations for its use in cell culture. As a result, the first step in the practical part of this project was to assess which concentration of NAC should be added to the SPM to better protect cells from etoposide without causing any extra damage.

Four different concentrations of NAC (5, 1, 0.5 and 0.05 mM) were tested, alone and in combination with 25 µg of etoposide. To evaluate which one was the best, we tested these conditions in 3 different semen samples (n=3) and decided according to these preliminary results. After incubation, we proceeded with the semen analysis followed by DNA analysis for each sample. The most relevant findings regarded the integrity of the spermatozoa. While evaluating the morphology of each sample after incubation (Figure VII-7A), we discovered that high concentrations of NAC were responsible for a decrease in the percentage of normal spermatozoa. In fact, we could clearly see that samples exposed to high doses of NAC had several spermatozoa with granulated heads and presented several tails with no head (Figure VII-7B).

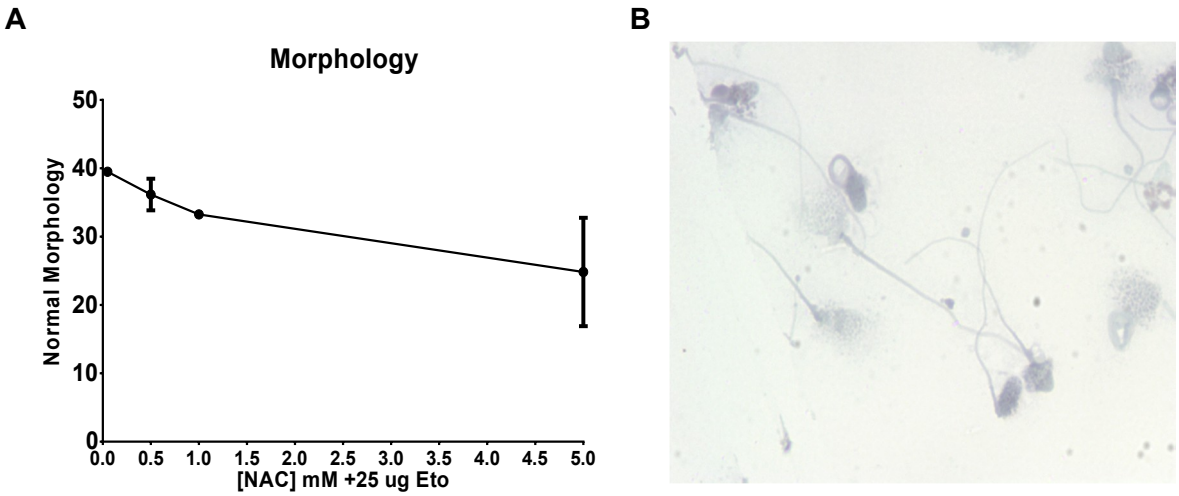

Figure VII-7. Alterations in spermatozoa morphology after exposure to increasing concentrations of NAC. (A) Percentage of normal spermatozoa with increasing doses of NAC; **(B)** Spermatozoa morphology after incubation with 5mM of NAC, total amplification 400X. Results are expressed as mean ± SEM (n=3 for each condition).

A 5 mM NAC concentration disrupted spermatozoa integrity, directly interfering with the viability of these cells. Therefore, we set this concentration aside and evaluated the capability of NAC 1, 0.5 and 0.05 mM to protect sperm DNA integrity when cells were exposed to etoposide (25 µg). Samples exposed to etoposide revealed more than 30% of dark blue cells, with uncondensed DNA, after AB staining (Figure VII-8A). These percentages were similar in the samples treated with 1 and 0.5 mM of NAC. However, spermatozoa incubated with etoposide and 0.05 mM of NAC showed a 13% decrease in the percentage of immature chromatin condensation. After incubation with etoposide, samples had a 32.5% of normal spermatozoa with fragmented DNA (Figure VII-8B). The supplementation with 1 mM of NAC did not alter this percentage. However, when samples were supplemented with 0.5 and 0.05 mM of NAC, there was a decreased in these levels to values lower than 20%.

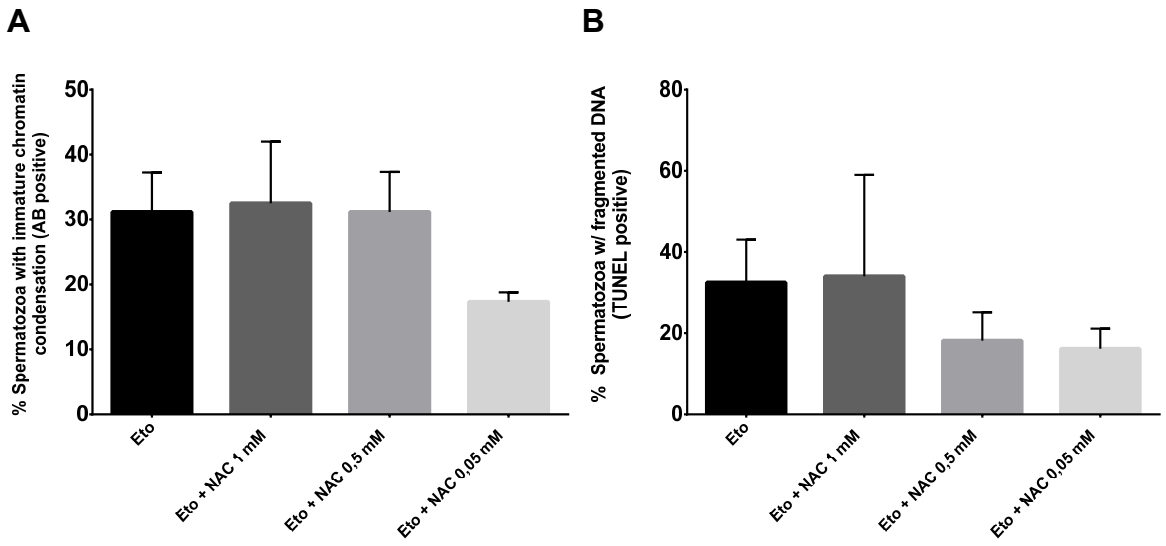

Figure VII-8. Spermatozoa DNA integrity when exposed to etoposide and supplemented with decreasing concentrations of NAC. (A) AB positive spermatozoa, in percentage, after incubation; (B) TUNEL positive spermatozoa, in percentage, after incubation. Results are expressed as mean ± SEM (n=3 for each condition).

These results led to the conclusion that a concentration of 0.05 mM of NAC would be the most appropriate to protect cells from etoposide, without interfering with spermatozoa integrity.

III. Semen Parameters Reference Values

Semen sample parameters need to be compared with reference values to allow correct patient diagnosis and management. Furthermore, threshold values are likewise important for clinical trials and scientific investigations. The reference values given have been generated from the results of several studies of semen quality and men fertility (Table VII-2).

Table VII-2. Lower reference limits (5th centiles and their 95% confidence intervals) for semen characteristics.

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)
pH	7.2

IV. Spermatozoa protein extraction

To identify the best protocol for protein extraction of human spermatozoa we tested several procedures in 12 semen samples and quantified the amount of protein that each one was able to extract from one million spermatozoa. We then compared the results obtained for each procedure and selected the best one to use with samples, including in the present research. The procedures tested varied on the lysis buffer used, times of incubation and the presence or absence of a sonication step. Figure VII-9 represents the different approaches tested and the main distinct steps between them.

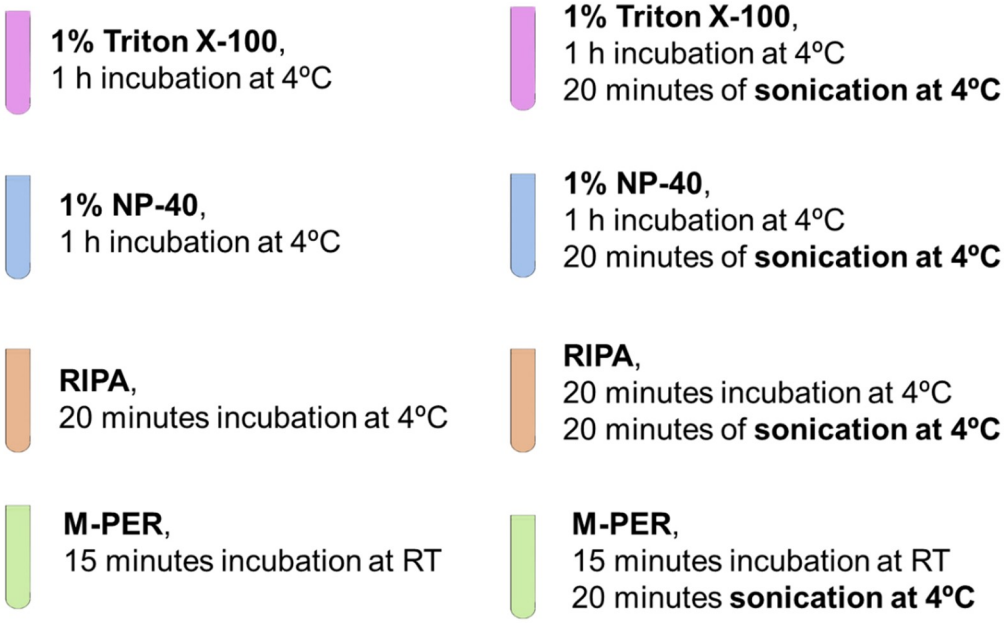

Figure VII-9. Schematic representation of spermatozoa protein extraction protocols. A total of 8 distinct approaches were tested. Spermatozoa were incubated in four different lysis buffer (1% Triton-X100, 1% Tergitol Type NP-40, Radio-Immunoprecipitation Assay buffer (RIPA) and Mammalian Protein Extraction Reagent (M-PER)) and then sonicated or not for 20 min at 4ºC.

At the end of each procedure, samples were quantified using the BCA Protein Assay Kit according to the manufacturer's instructions. To compare the different protocols we evaluated the quantity of protein extracted from one million spermatozoa (Figure VII-10). Although results were not significant, except for Triton X-100 with sonication and Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL, USA) ($p=0.001$), the protocol with 1% Triton X-100 lysis buffer and 20 min sonication displayed the best outcomes. This condition had the highest mean of protein extracted (11 µg/µl) and

displayed more consistency of high results than all other protocols, without restricting amounts for latter protein use.

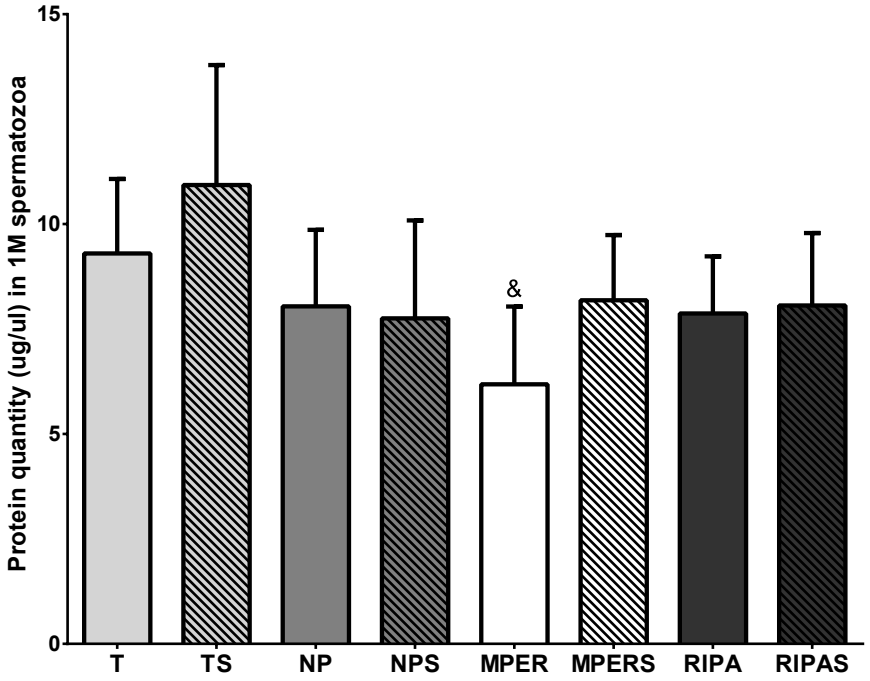

Figure VII-10. Protein extracted in µg/µl per one million spermatozoa after each procedure. Median of protein quantity extracted following each protocol. TS group displayed the higher median in comparison to the remaining procedures. Abbreviations: T, Triton X-100; TS, Triton X-100 with sonication; NP, NP-40; NPS, NP-40 with sonication; MPERS, MPER with sonication; RIPAS, RIPA with sonication. Statistical analysis was determined by one-way ANOVA, followed by Fisher's LSD. Results are expressed as mean ± SEM (n = 12 for each condition). Significantly different results ($p < 0.05$) are indicated as: & - relative to TS group.

Spermatozoa protein extraction: Selected protocol

1% Triton X-100 with sonication

Procedure:

1. Lysis buffer (1% Triton X-100, 2M Tris buffer (Sigma), supplemented with 1% protease inhibitor cocktail (PIC; Thermo Fisher Scientific, Waltham, MA, USA) and 100 mM sodium orthovanadate (phosphatase inhibitor; Sigma) was added to the sperm pellet;
2. Incubated for 1 h at 4°C, while gently shaken;
3. Sonicated for 20 min at 4°C;
4. Centrifuged at 14000 xg for 20 min at RT;
5. The resulting pellet was discarded and supernatants were reserved at -20ºC.